LEVELS OF ENDOGENOUS GROWTH REGULATORS OF GRAPEFRUIT (*Citrus paradisi* Macdf.) DURING FRUIT SET

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1980

To my family, whose constant encouragement and love made it all possible.

ACKNOWLEDGMENTS

The author expresses his profound gratitude to Dr. J. Soule, chairman of the supervisory committee, for his invaluable advice and suggestions during the preparation of the manuscript.

The author is particularly indebted to Dr. T.A. Wheaton, co-chairman of the supervisory committee, for his technical advice, guidance and more than adequate availablility of time and laboratory facilities throughout the study.

Deep appreciation is expressed to Dr. A.H. Krezdorn, Professor Emeritus and former chairman of the supervisory committee for suggesting this problem and for his constant support, constructive criticism and guidance during the execution of the research and the preparation of the manuscript.

Appreciation is extended to Dr. R.H. Biggs and T.E. Humphreys for their literary advice and to Mr. A.J. Rose, of Libby, McNeil and Libby Corporation, for providing the trees used in this research.

The invaluable assistance of Mr. Steven Hiss, Mr. Kazuhide Kawada, Mr. Paul Rebenack, and Mrs. Jane Van Clief

is acknowledged and greatly appreciated. Special thanks go to Mrs. Peggy Lee for typing the manuscript.

The author acknowledges the economic support by the Agricultural Research Corporation of the Sudan during most of this research and the financial assistance provided by both the Fruit Crops Department and the Agricultural Research and Education Center, Lake Alfred, towards the end of this study.

Finally, the unlimited support, endurance and devotion of the author's wife, Widad, is warmly acknowledged.

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August 1980

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Major Department: Horticultural Science (Fruit Crops)

The various events in the sexual process and the endogenous levels of IAA (indoleacetic acid), GA (gibberellic acid) and ABA (abscisic acid) for seedy 'Duncan' and seedless (parthenocarpic) 'Marsh' grapefruit (*C. paradisi* Macdf.) from anthesis through early seed development were investigated. Pollen grains germinated almost immediately on 'Duncan' stigmatic surfaces; pollen tubes reached the ovules in 8 and 6 days in 1978 and 1979, respectively. Fertilization occurred 4 days later in both seasons. Free nuclear endosperm was observed 12 and 10 days after anthesis in 1978 and 1979, respectively. Germinating pollen grains or pollen tubes were rarely observed in 'Marsh' styles.

Degeneration of ovules was noted from anthesis to termination of sampling.

Levels of growth regulators found in young 'Duncan' and 'Marsh' fruit were 21-766 ng IAA, 13-395 ng ABA and 0.062-0.435 ng GA/g fresh weight. These growth substances followed a similar pattern in both cultivars and no meaningful differences in their levels were observed. Thus, the ability of the parthenocarpic 'Marsh' fruit to produce these growth substances equally as well as the seedy 'Duncan,' was evident.

Pollination and pollen tube growth coincided with large increases of IAA, GA and ABA. Levels of IAA and GA in both seasons and ABA in 1979 decreased in the period shortly before fertilization and continued to decrease for about 40 days thereafter, at which time they leveled off. GA, on the other hand, produced a second peak about 14 days after fertilization. Whether the peaks of growth regulators in 'Duncan' resulted from the events of the sexual process that coincided with or followed them is not certain.

INTRODUCTION

The roles of pollination, pollen tube germination and growth, sexual fertilization and seed development upon fruit set and development are well documented (21, 22, 54, 81, 83, 186, 187). Parthenocarpic production of seedless or few-seeded citrus fruit, however, is characteristic of several important cultivars. Seedless cultivars are much more desirable than seedy ones when fruit is marketed fresh.

The dependence of fruit set and development upon growth regulators, both for seedy and seedless fruit, has been postulated (36, 44, 67, 70, 71, 72, 73, 124, 149, 150, 152). Early work suggested that auxins produced in seeds control fruit set and that parthenocarpic fruit set was a result of a higher auxin content in ovaries of certain cultivars (72). More recent work suggests that other growth substances are also involved (40, 124, 127, 130, 151); however, the biochemistry of fruit set and development is incompletely known. Much of the early work was hampered by inadequate procedures for qualitative and quantitative identification of growth regulators. Improved methodology has increased the likelihood of more

precisely determining their relation to fruit set and development.

Evidence that growth regulators are involved in fruit set and development is strengthened by a number of instances where fruit set is improved by exogenous application of growth regulators (26, 37, 41, 89, 106, 109, 110, 154, 178, 180, 189, 196, 197, 198, 204).

Research was, therefore, initiated to determine the relationship of endogenous growth regulators of seedy and seedless (parthenocarpic) grapefruit cultivars to fruit set and development through June drop.

LITERATURE REVIEW

Pre-Anthesis Development of Flowers

Seasonal conditions may cause citrus to bloom at various times; however, the main flowering period in humid subtropical climates is the spring. Irregular bloom "off bloom" is occasionally caused by several factors such as rainfall or heavy irrigation preceded by a dry period (2, 202). In the tropics or dry subtropics, on the other hand, citrus tends to flower with the onset of rain following dry weather.

Two types of bloom are common in citrus, "leafy" and leafless or "bouquet" bloom. Both occur on the tree at the same time, but flowers on leafless shoots open about 1 week earlier. In general, more fruit are set on leafy inflorescences than on leafless ones (13, 174).

The time of flower-bud differentiation in 'Duncan' grapefruit, 'Pineapple' sweet orange (C. sinensis [L.] Osbeck) and 'Owari' satsuma (C. unshiu Marco.) occurs at the initiation of growth in the spring or at any other season of the year after a sufficient period for the accumulation of reserve food (2). 'Valencia' sweet orange and sweet lime (C. limettioides Tan.) flower-bud

initiation reportedly exhibit the same pattern (165). Flower-bud induction of 'Shamouti' orange was observed in November and December (6). Although citrus trees usually bloom heavily, many flowers and young fruit drop before reaching maturity. A multitude of climatic, nutritional, disease and other physiological factors were reported as primary causes of fruit drop (9, 11, 24, 51, 52, 75, 79, 88, 99).

The megasporogenesis process in plants has been well investigated by several authors (7, 132, 147, 155). The 'Washington' navel sweet orange and satsuma mandarin (155), 'Foster' grapefruit and 'Pineapple' sweet orange (7, 147) embryo sacs were reported to mature at different periods.

The Sexual Process

Pollination and Pollen Tube Growth

The relation between pollination, fertilization and fruit set in citrus has been investigated over the years by many workers (1, 15, 22, 57, 58, 154, 169, 177, 179, 186, 187). Most citrus cultivars are self- and cross-compatible. Sexual incompatibility and gametic sterility, however, are not uncommon. 'Clementine' tangerine (C. reticulata Blanco) (154, 177), 'Orlando' tangelo (C. reticulata x C. paradisi) (105, 108), 'Minneola'

tangelo (105, 108), 'Robinson,' 'Lee,' 'Osceola,' 'Nova' and 'Page' mandarin hybrids [C. reticulata x C. (reticulata x paradisi)] (169, 201), have all been reported to be either self- or cross-incompatible. On the other hand, degeneration of both the ovule and pollen was observed in 'Marsh' and 'Thompson' grapefruit (58, 90). Other reports described also complete gametic sterility in 'Washington' navel orange (58, 155).

Pollen germination and pollen tube growth in citrus has been described by several workers (21, 22, 177, 179, 186, 187, 207). Environmental conditions, such as temperature, and other related factors were reported to affect pollen tube germination and growth (17, 46, 47, 141). Inhibition sites causing unsatisfactory germination and pollen tube growth were reported in the stigma (116, 117), style (54), and ovary (135). Following pollination, the pollen grew downward the style and eventually reached the ovary, ovule and embryo sac in some citrus cultivars (21, 22, 177, 179, 186, 187).

The time required for the pollen tube to reach the ovule varies with cultivars. 'Duncan' pollen tubes were reported to penetrate the embryo sac of 'Orlando' tangelo 4 and 12 days after pollination in 1963 and 1962, respectively (21). Ton and Krezdorn (187) showed pollen tubes of 3 incompatible citrus cultivars reached the ovule 5 to 8 days after pollination. Oppenheimer

(154) found pollen tube fragments of 'Duncan' grapefruit in the lower portion of the style of 'Clementine' tangerine within 3 days and near the ovules within 6 days. Other reports also indicated the presence of pollen tubes in the ovary and the ovules in periods ranging from 6 to 8 days after pollination in several citrus cultivars (206, 211).

The pollen tube transfers 2 sperm nuclei to the embryo sac in the ovule. This process is required in several citrus cultivars and other fruit crops for successful fertilization and subsequent fruit set (7, 10, 58, 107, 125, 148, 149). Many fruit, however, are set parthenocarpically (i.e. without fertilization). The degree of parthenocarpic fruiting varies among citrus cultivars and other fruit crops (31, 58, 153, 201). Several workers have shown that the growth of the pollen tube downward through the style may cause a "primary stimulus' that triggers the fruit to set and develop (70, 71, 131, 184, 208). Thimann (184) and Gustafson (71) supported the "primary stimulus" theory by suggesting the possibility of stimulating the growth of ovaries into mature seedless fruits with pollen extracts. The latter were postulated to contain growth hormones.

Fertilization and Subsequent Process

The time for completion of fertilization in citrus differs with cultivars. Fertilization in satsuma was accomplished in 30 minutes in contrast to 4 weeks in trifoliate orange (P. trifoliata [L.] Raf.) (29). Strasburger and Toxopeus, cited by Frost (56), found that fertilization in several citrus cultivars occurred 3 or 4 weeks after pollination. According to Coit (30), Ideka found that fertilization occurred about 48 to 72 hours after pollination; however, the species used were not mentioned. Since the actual fertilization process had not been observed, other reports (7, 10, 21, 83) indicated variable time estimates for the occurrence of the process.

The endosperm nucleus undergoes successive divisions to produce a multinucleate endosperm a few days after fertilization. Bacchi (7) observed about 1,500 nuclei within 1 endosperm in 'Foster' grapefruit. The nuclear stage of the endosperm he observed continued through the 67-day sampling period. Banjeri (10), Carlos (21), and Hensz (83) also reported multinucleate endosperm stages in other citrus cultivars that shrank during embryo development and completely disappeared 100 days after pollination.

The zygote enters a resting period for several weeks before dividing (7, 21, 155). This period ranges

from 21 to 28 days in trifoliate orange, while it lasts 50 days in 'Foster' grapefruit. An 'Orlando' tangelo zygote undergoes nuclear division 40 days after pollination (21). Subsequent divisions produce the cotyledonous lobes, the dermatogen, periblem and plerome. The 4-celled suspensor can be clearly defined 5 days after the first zygotic division (21, 58).

Polyembryonic cultivars in citrus are common (10, 29, 56, 133, 155, 188, 199, 200). Nucellar embryos may develop on stalks attached to the nucellus before the division of the egg cell. They are distinguished by their irregular shape and great variety in size, as well as by the absence of a suspensor (155). Both the endosperm and nucellus later shrink leaving only small fragments that contribute to the formation of the inner seed coat (55, 58).

Growth and development of citrus fruit are well documented in the literature (8, 11, 14, 21, 50, 86, 175). Fruit development follows a simple sigmoid curve, 3 characteristic stages of 'Valencia' sweet orange being described by Bain (8). Stage 1, which lasted from 4 to 9 weeks after full bloom, was designated by both a high metabolic activity and rate of cell division. Increase in fruit size at this stage was due mainly to growth in central axis, septa, flesh and peel. Stage 2 was primarily a period of maximum fruit growth. The marked

expansion of tissues in this stage was dominantly due to rapid cell enlargement and differentiation. Expansion due to cell division was very limited and continued in the outer peel tissue. The pulp expanded considerably, juice vesicles became larger and juice content increased. Stage 2 lasted approximately 29 weeks. Stage 3, the maturation period, lasted approximately 28 weeks. It was characterized by a slower rate of fruit growth. Maturity changes occurred during this period.

The Role of Growth Substances in Fruit Set

Numerous attempts have been made in recent years to investigate the physiology and nature of fruit set in plants (36, 72, 100, 106, 149). Both parthenocarpic and non-parthenocarpic cultivars have been utilized to elucidate the hormonal basis and need of the sexual process during the early stages of reproduction (21, 70, 71, 72, 81, 83). The theories and hypotheses advanced generally involved carbohydrates and a hormonal regulatory mechanism. The complexity of this mechanism and lack of appropriate analytical techniques have hampered any rapid progress in this field (36, 103, 203).

Auxins

Auxins are an essential part of the hormonal system in plants. Their significant role in fruit set has been

discussed by several workers (36, 70, 71, 72, 81, 113, 118, 150, 181, 191). The presence of auxins in pollen, its production in the style, ovary and ovule accompanying pollen tube growth and fertilization, and the resultant stimulation in growth of the ovary are well established facts (150, 191). An early hypothesis attributed parthenocarpic fruit set to the higher auxin content in the ovaries of seedless cultivars at the time of blossoming. Development of the ovary in seedy cultivars, which require pollination and fertilization, was postulated as being due to the auxin produced in developing seeds (72). The importance of auxin in this respect was shown by the increase of fruit set of a number of species with exogenous application of auxin (36, 113). Later research, however, refuted this idea by showing that the auxin content of the seeds was correlated with seed development but not with fruit growth (5, 33, 40, 74, 124, 127, 128, 149, 206), and that normal fruit development in parthenocarpic cultivars might not be achieved with exogenous auxin application. Involvement of other growth hormones in this respect seemed to be essential (156, 162). Other workers (129, 130, 144, 145) have suggested that the amount of auxin in the pollen would not be sufficient to cause ovary growth. They postulated that auxin might be released from a precursor by an enzymatic system supplied by the pollen. Muir (144, 145) found more auxin

in pollen tubes than in pollen alone. He also detected an enzyme system that could release auxin from its bound form and an increase in the auxin content of the pistil following pollination.

The importance of the endosperm as a primary source of auxin in the seed has been stressed in the literature (40, 124, 126, 146). An auxin build-up in developing apple seeds was observed coinciding with the development of the endosperm. A variation in auxin production could be correlated with 3 periods of fruit drop (126).

Correlations have been reported between auxin level increases in newly-set fruit and fruit set in studies with citrus (81, 118). A remarkable peak of auxin production in young 'Washington' navel orange fruit was observed soon after full bloom and a second one occurred at the beginning of the cell enlargement phase of fruit growth (118). The same sequence was found in 'Orlando' tangelo, 'Parson Brown' and 'Dream' navel oranges, along with a third peak during the cell enlargement phase (81). Takahashi et al. (183) reported significant changes in auxin content in the developing C. unshiu fruit. Two definite peaks occurred within 5-7 and 30-35 days respectively, and a minimum peak was observed 20 days after full bloom. Monselise et al. (142),

in contrast, did not detect any promotive auxin activity when they traced seasonal changes in developing 'Shamouti' orange fruit.

Fruit have been shown to be the organ into which nutrients flow often at the expense of other plant parts (4, 35, 36, 112, 113, 119, 120, 122, 123, 137, 182). Marre and Murneek (134) demonstrated the mobilizing action of auxin on the movement of other substances, i.e. carbohydrate and minerals, into reproductive structures. Similar investigations revealed the importance of auxin as a mobilizing factor (77, 104, 122).

There is considerable evidence that growth promoters other than auxin are involved in regulating fruit set and growth (28, 33, 41, 42, 59, 131, 157, 162). The effectiveness of gibberellins in setting fruit which did not respond to auxin treatments suggested such a hormonal regulatory system (26, 41, 84, 85, 110).

Gibberellins

The capacity of gibberellins for setting different types of fruit has been elucidated comparatively recently (26, 41, 42, 84, 110, 163, 180). Figs (42), pears (68), and apples (20, 43, 44) responded with varying degrees to auxin but their fruit could be set equally well or better with GA. Auxins have been ineffective in setting citrus fruit, but GA significantly increased set in the

self-incompatible 'Clementine' mandarin (180), 'Orlando' tangelo (105, 108) and the 'Washington' navel orange (84). Tree damage and lower fruit quality due to GA application were also observed in citrus cultivars (27, 84, 180). The response of citrus fruit to GA application, however, varied with concentration, method and time of application (27, 28, 84). Other fruits, e.g., cherry, required both GA and auxin application for parthenocarpic fruiting (36).

The presence of naturally occurring gibberellins in seeds of higher plants (103, 106, 160) is suggestive of an active role for these substances in fruit set, growth and development. Stimulation of growth in plants by either cell division and/or cell enlargement has been related to gibberellins (12, 16, 19, 76, 121, 170, 171, 205). It has been proposed that gibberellins stimulate growth by interaction with auxin (97, 111, 157, 159, 172, 173). It may be inferred from this concept that gibberellins regulate fruit set and development through increasing endogenous levels of auxin. Previous research has demonstrated the validity of such inference using both vegetative tissues of plants and tomato fruit (97, 111, 159, 172). Sastry and Muir (172) contended that pollen-gibberellins released at pollination caused an increase in the level of diffusible auxin in the tomato ovary. Other reports refuted this concept since many fruits

including apples failed to produce auxin several weeks after anthesis (36).

Correlations have been reported between endogenous gibberellins, fruit set and growth patterns (25, 176). Results that related fruit growth and levels of gibberellins, however, were not consistent. Isolation of GA_1, from water sprouts of C. unshiu was one of the first identifications of gibberellins in tissues of higher plants (96). Khalifah et al. (102) found 3 gibberellin-like substances in young orange and lemon fruit, 2 of which were tentatively identified as GA_1 and GA_9. The presence of at least 3 gibberellin-like compounds has been recently described in the flavedo of maturing orange fruit (62). A build up of gibberellin-like substances prior to and during the regreening of 'Valencia' oranges was noticed (168). Erner, Goren and Monselise (53) found that both the flavedo and albedo of rough 'Shamouti' orange fruit contained much more gibberellin-like substances than the corresponding tissues of smooth-peeled fruit. Significant changes in gibberellins were shown to occur both in tissue concentrations and total amount per fruit in samples collected during the initial stages of fruit development. These findings were interpreted as suggesting a "cause and effect" relationship between certain gibberellins and fruit growth (205). Native gibberellins have also been reported in seeds,

fruit and other tissues of apples, banana, apricot, vegetable and field crops (24, 45, 82, 95, 100, 143).

The ability of gibberellins to increase the photosynthetic rate and the mobilization of metabolites to actively growing plant parts has been demonstrated (38, 77, 80, 158). Work by Crane (36) has shown that pollination fertilization, high promoter content or application of gibberellins promoted fruit set by creating a gradient of high metabolic activity in the ovary. The flow of metabolites from other plant parts to the ovary would therefore prevent its abscission. Research in support of this theory revealed that metabolites were actively translocated to developing embryos and fruits (77, 119, 182) and fruit abscission might be controlled by auxin movement from other plant parts to the fruit (60, 130, 209, 210). Furthermore, Powell (161) demonstrated the mobilization of leaf metabolites into young citrus fruits with gibberellin applications.

Cytokinins

The exact nature of citrus cytokinins has never been determined and published data are relatively scarce. Research, however, has shown the importance of cytokinins together with gibberellins and auxins in the regulation of cell division, cell enlargement, differentiation and organogenesis in developing plants (12,16, 19, 76, 138,

192). Van Overbeek (192) suggested that cytokinins might be involved in the early stages of fruit growth, earlier than those affected by gibberellins. Cytokinin-like substances have been found in more than 40 plant species (115). Active extracts were obtained from peach, pear, apple, quince, plum and tomato fruits (115). Khalifah and Lewis (101) detected cytokinin activity in lemon seed extract in their pioneer work. Determinations of cytokinins in rough and smooth 'Shamouti' orange fruit showed more of these substances in the rough peel. Also, a higher content was found in flavedo as compared to albedo tissue (53). The existence of cytokinin activity in extracts of other plant parts has also been mentioned in the literature (51).

Effects of exogenous application of cytokinins on fruit set and development have been studied. These effects involved increase of fruit set in muskmelon and grape (97, 196, 197), promotion of parthenocarpic fruit set in figs and some apple cultivars (39, 136) and stimulation of fruit growth in grapes. A close correlation between cytokinin, auxin and berry development was established in the latter case (197).

The mobilization capacity of cytokinin has been investigated using both senescing leaves and plant reproductive parts (36, 69, 113, 114, 115). The flowering and fruiting process have received lesser and limited

research in this regard. Application of N-6-benzyladenine (BA), a synthetic cytokinin, at anthesis to young fruit of muskmelon has increased fruit set (94). The fact that a cytokinin application increased DNA, RNA, protein synthesis and mobilized metabolites to the point of application was hypothesized as a reasonable cause for increasing the competitive ability of the treated fruits and hence to increase fruit set (94, 193).

Abscisic Acid

The existence of a β-inhibitor complex of aromatic acids with different properties and compositions was revealed in apricot, lemon and strawberry fruits (140, 185, 195). ABA was detected in lemon juice by the optical rotary dispersion technique soon after its identification as the principal component of the β-inhibitor (140). Little is known with regard to ABA's role in fruit set but Thompson (185) suggested that growth of strawberry fruit tissues (carpels) ceased at anthesis because of an inhibitory factor produced by the carpels. Pollination and fertilization would possibly overcome this inhibition. This hypothesis was refuted by Wright (206), who detected an acid inhibitor 18 days before fertilization in black currant (Ribes nigrum) fruit. It progressively increased in concentration until 17 days after fertilization.

Monselise et al. (142) reported the presence of large amounts of inhibitors in developing fruit and mature leaves of 'Shamouti' orange. Their report was supported by the studies of Goldschmidt et al. (65, 66), who showed the presence of ABA-like inhibitors in citrus leaves and branches and an increase in the amount of inhibitors during fruit growth. Recent work in Japan indicated that ABA in the young satsuma fruit has 2 maximum peaks. The first one was 7 days after full bloom, sharply decreased to a minimum content 18 days later, and the second one was attained 43 days after full bloom. The content of ABA thereafter increased steadily but slowly (183).

ABA concentrations in mature fruit peel are among the highest reported in the literature. Pigmented plant tissues were noticed to contain more growth inhibitors than pigmentless ones (98). In accordance with this view, the pigmented flavedo was found to contain more inhibitors than the albedo (64, 67, 166, 167), and the pigmentless petals have low inhibitor contents (61). Recent work, however, contradicted these findings and showed relatively similar amounts of ABA in both albedo and flavedo tissues (18). Accumulation of ABA in fruit tissues close to maturation has been shown in several plant species (63, 98). Hoad (87) has determined the endogenous ABA levels in the fruit, leaves, and fruit phloem exudate of Lupinus alba L. as affected by water stress.

Increase of fruit set has been obtained in apples (139) and grapes (32) with pre- and post-bloom applications of growth retardants. Application of ABA usually causes abscission of flower parts and young fruits, however, hence its effect on citrus fruit set is inhibitory rather than promotive (198). Treatment of 'Temple' orange with ABA hastens coloring and onset of senescence (34). Acceleration of abscission, inhibition of growth and flowering, and induction of a rest period are all biological effects involving ABA treatment (3, 198).

Hormonal control of fruit set and development continues to be a central, greatly unresolved problem. The best evidence, however, appears to indicate a critically balanced hormonal mechanism involving auxins, gibberellins, cytokinins and possible other inhibitory substances regulates fruit set and development. Closely related to such a hormonal balance are enviromental factors and other physiological processes.

MATERIALS AND METHODS

Experiments were conducted with 22-year-old 'Marsh' and 'Duncan' grapefruit trees on rough lemon (C. jambhiri Lush.) rootstock. 'Marsh' is both male and female sterile but strongly parthenocarpic, thereby producing fruit that are few-seeded (0-6 seeds per fruit). 'Duncan' is an extremely seedy cultivar (60-70 seeds per fruit) and non-parthenocarpic. Trees were grown on an Astatula fine sand in Lake County and managed in a standard commercial manner.

Thirty and 20 trees of each cultivar with good bloom were selected in 1978 and 1979, respectively. Sufficient leafy bloom flowers were thinned and tagged for proper identification just prior and during anthesis in both seasons. Tagged flowers were located around the outer canopy of each tree from 1 to 2.5 m above the ground.

Pistil, ovary and young fruit samples were collected at 2-day intervals during the early stages of fruit growth and thereafter as indicated in the schedule in Table 1. Samples were collected between 9:00 A.M. and 1:00 P.M.

Table 1. Schedule for sampling ovaries and young fruit of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1978, 1979.

Season[z]					
1978			1979		
Mar.	29	(-2)	Apr.	2	(-2)
	31	(Anthesis)		4	(Anthesis)
Apr.	2	(+2)		6	(+2)
	5	(+4)		8	(+4)
	7	(+6; +8) (PF)		10	(+6)
	10	(+10)		14	(+8; +10) (PF)
	12	(+12)		21	(+12; +14; +16)
	15	(+14)		24	(+18; +20)
	18	(+16; +18)	May	1	(+27)
	22	(+20)		10	(+34)
	29	(+27)		16	(+41)
May	6	(+34)		25	(+48)
	13	(+41)		29	(+55)
	19	(+48)	June	12	(+64)
	28	(+55)		26	(+85)
June	6	(+64)	July	3	(+99)
	22	(+85)			
July	7	(+99)			

[z]Days before (-) or after (+) anthesis; petal fall (PF).

Parthenocarpic Status of Cultivars

Two trees of each cultivar were used in the 1978 season. Sixty flowering shoots of new growth were thinned to 1 large terminal flower, which in turn was depetaled, emasculated and treated. Treatments consisted of self- and cross-pollination using both 'Duncan' and 'Marsh' grapefruit pollen. Thirty flowers were used for each treatment in each tree.

Fruit Growth Measurements and Drop

Four representative young fruit from each tree were removed and composited at each sampling date (Table 1). Fresh weight and equatorial diameter were determined using 50 fruit randomly selected from each composited sample. Percent growth rate of fruit was estimated from the fresh weight data.

Fruit drop counts from full bloom through June drop were made from 6 trees in the 1979 season. Fruit drop estimates were obtained at each sampling date by counting the fruit caught in saran cloth-covered frames placed under the canopy of each tree, and from periodic counts of fruits on 4 tagged branches on each tree. The percentages of fruit falling between each sampling date were determined from the counts of fruits from the 4 tagged branches.

Anatomical Studies

Pollen Tube Growth and Seed Development

Sets of 15 pistils, ovaries and young fruit were collected and killed and fixed in 50% FAA (90 ml 50% EtOH (ethanol): 5 ml glacial acetic acid : 5 ml formalin). Young fruit were trimmed along 2 sides to facilitate FAA penetration at each sampling date.

Microscopic observations were made of the pollen tube growth using the squash technique described by Yap (207) and modified by Hensz (83).

Progress of pollen tube in the ovule, fertilization and the subsequent seed and fruit development were observed in paraffin sections. Ovaries, young fruit and seeds were aspirated for at least 12 hours, dehydrated, embedded in paraffin, sectioned at 5-15 µm with a rotary microtome, and double stained with safranin plus fast green FCF (91, 92, 93) or pontacyl violet 6R (48).

Growth Substances Studies

Fruit samples were frozen immediately after collection by placing them into plastic bags immersed in a cold dry ice-acetone bath. Bags were placed in a chest with dry ice during transportation to Gainesville and held at -18°C until extraction.

Extraction and Identification

Extraction and purification of IAA, ABA and GA were based on the method described by Wheaton and Bausher (203). Duplicate samples (10 g fresh weight) of ovaries, whole young fruit and fruit sections were used as the season progressed. Samples were put into boiling ethanol for enzyme inactivation, chilled in an ice bath, homogenized, re-extracted and filtered. Isotope-labelled ABA and IAA (1×10^3 dpm, each), (DL-cis-trans-[2-^{14}C] abscisic acid) (11.3 mCi/mmol) and 3-Indolyl [1-^{14}C] acetic acid (57.6 mCi/mmol), (Amersham) standard was added in the first extraction step for recovery determination.

Ion-exchange chromatography was used for separation of the sample into acidic, basic and neutral fractions. The technique was adapted from that described by Raj and Hutzinger (164) for acidic compound recovery (DEAE Sephadix; acetate form) and by Van Staden (194) for recovery of cytokinins (Dowex 50, hydrogen form).

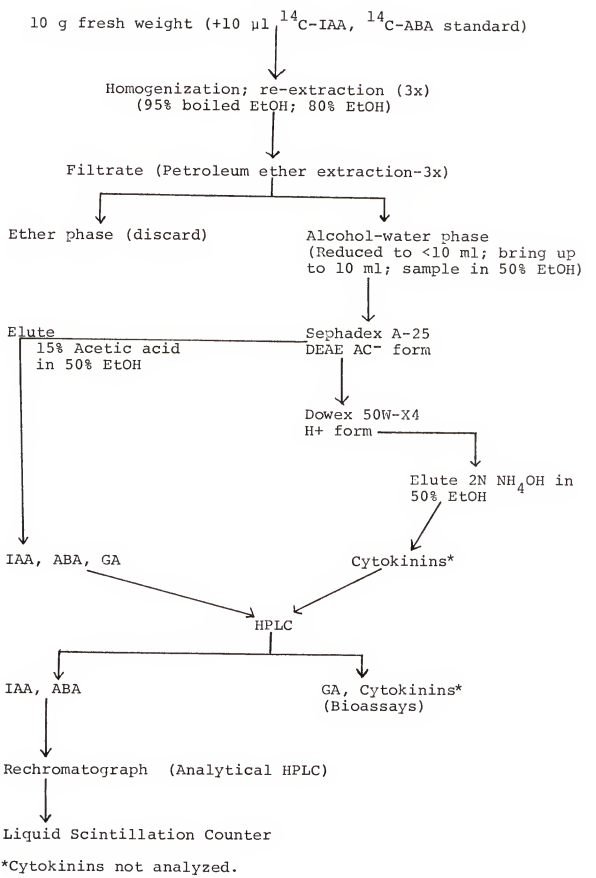
A modular High-Performance Liquid Chromatography (HPLC) system with a Waters-μ-Bondapak C-18 (10 μm; 30 cm (L) x 3.9 mm (ID) or RP-8 (10 μm; 25 cm (L) x 4.6 mm (ID)) (Lichrosorb) reverse phase columns was used for further purification of the extracts. Each sample was eluted with a linear gradient of ethanol (0-50% in 50 min.) in 0.5% ammonium acetate (pH 5.1), delivered by

2 constant flow, high pressure Waters M-6000 pumps and controlled by a solvent programmer (Waters Associates, model 660). Flow rate was 2.0 ml/min. Effluent from the column was monitored directly by UV (254 nm) (LDC 1200 spectroMonitor I) or a fluorescent detector (excitation 290 nm; emission 360 nm) (Aminco-Bowman spectrophoto fluorometer). Effluent fractions containing IAA, ABA, and GA were collected based upon the retention time of known volumes of the respective standards (Sigma Chemical Company). IAA and ABA fractions were further rechromatographed isocratically using a solvent system of 2.5% or 12.5% ethanol, in 0.2% ammonimum acetate (pH 6.5), respectively. Effluents from the column were monitored either by using a Schoeffel SF/GM 770 variable wavelength detector for absorbance measurements or the fluorescent detector described previously. Radioactivity in the IAA and ABA fractions collected was counted ina liquid scintillation counter (Beckman, LS-100 model). A diagram of the analytical procedure is given in Figure 1.

Quantitative determination of GA utilized a modification of the barley endosperm bioassay described by Van Onckelsen *et al*. (190). Seeds of Himalaya barley (Agronomy and Soils Department, Washington State University) were cut transversely and the embryo portion was discarded. GA fractions obtained from the HPLC were reduced to

dryness and 3 ml calcium chloride ($CaCl_2$) buffer (0.2% W/V) were added. Each fraction was divided into 2 equal volumes in petri dishes and 10 µl of standard GA_3 (10^{-8} g/ml) were added to 1 dish for recovery determination. Petri dishes containing 5-half seeds each were wrapped in a black cloth and incubated at 30°C for 26 hours. Seeds were then homogenized in 10 ml $CaCl_2$ and the extracts were centrifuged for 20 minutes at 5,600 rpm. The resulting supernatant fractions were assayed for α-amylase activity using an automated procedure described by Harms and Camfield (78).

Fig. 1. Diagram of overall analytical procedure for growth substances.



*Cytokinins not analyzed.

RESULTS AND DISCUSSION

Parthenocarpic Status of Cultivars Used

'Marsh' set equally well when pollinated with its own sterile pollen as when pollinated with viable 'Duncan' pollen. This confirms the strong parthenocarpy of 'Marsh' that had been previously reported (58, 90). The fruit had only an occasional seed in both cases. 'Duncan' set well when self-pollinated but no fruit were produced when cross-pollinated with sterile 'Marsh' pollen (Table 2). Thus, Duncan is not parthenocarpic. These points were established in order to better interpret endogenous growth substances data.

Fruit Development

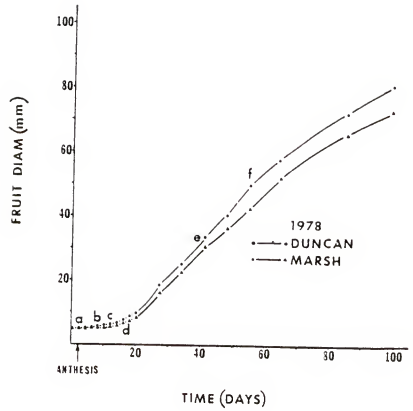
The growth curves of both 'Marsh' and 'Duncan,' based on equatorial diameter measurements of fruit, were those of the first, second and early third stage of a sigmoid growth curve (Fig. 2). This agrees with numerous reports on the growth of several species of citrus fruits (8, 11, 14, 21, 50). Stages 1 and 2 lasted approximately 20 and 65 days, respectively. 'Duncan' produced slightly larger fruits than 'Marsh' in both seasons.

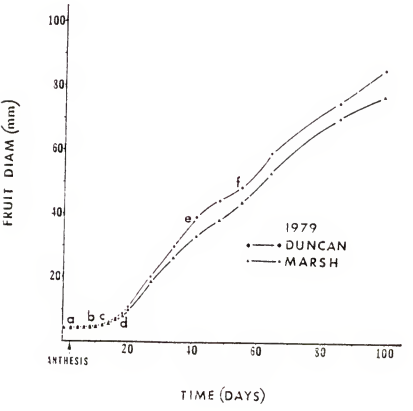
Table 2. Number and percentage of self- and cross-pollinated ovaries that abscised between the early sampling days in 1978.

Days after anthesis	'Duncan'				'Marsh'			
	Self		Cross		Self		Cross	
	No.[z]	%	No.[z]	%	No.[z]	%	No.[z]	%
Anthesis	0	--	0	--	0	--	0	--
+6	5	8	26	43	6	10	5	8
+12	12	20	60	100	10	17	14	23

[z]Each figure is a total from 2 trees of each cultivar; 30 "leafy" bloom flowers were used per treatment.

Figure 2. Fruit diameter of 'Duncan' and 'Marsh' grapefruit during the early growth period of ovaries and young fruit, 1978, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


FRUIT DIAM (mm)
100
80
60
40
20
a
b
c
d
e
f
1978
DUNCAN
MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)


FRUIT DIAM (mm)
100
80
60
40
20
a
b
c
d
e
f
1979
DUNCAN
MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Both 'Duncan' and 'Marsh' fruit had 4 peaks of growth rate, expressed as percent of daily change in fruit fresh weight, in 1978 (Fig. 3, 4). In 1979, 'Duncan' had 3 growth peaks and 'Marsh' had 5 (Fig. 5, 6). Peaks of fruit growth occurred in both seasons during the early period of active cell division and enlargement i.e. Stage 1, 2. Subsequent growth increments showed diminishing rates in the later stages of fruit development.

Inconsistency in time and number of growth peaks of 'Orlando' tangelo, 'Washington' navel orange and 'Parson Brown' fruit have also been reported (81, 205). Bartholomew (11) reported such fluctuations in his monthly measurements of growth of lemon fruit. He attributed this to moisture stress in the fruit during periods of spring drought. Climatic data were not collected during this period of this study but it is very likely that the fluctuations in fruit growth were related to soil moisture fluctuations.

Fruit Drop

Estimations of peak periods of fruit drop by both frame count and twig count methods were in general agreement (Table 3). Both bouquet and leafy bloom were included in the determination of peak fruit drop periods, while only leafy bloom were used in the anatomical and growth substances studies.

Figure 3. Mean daily percent growth rate of 'Duncan' grapefruit during the early fruit growth period, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

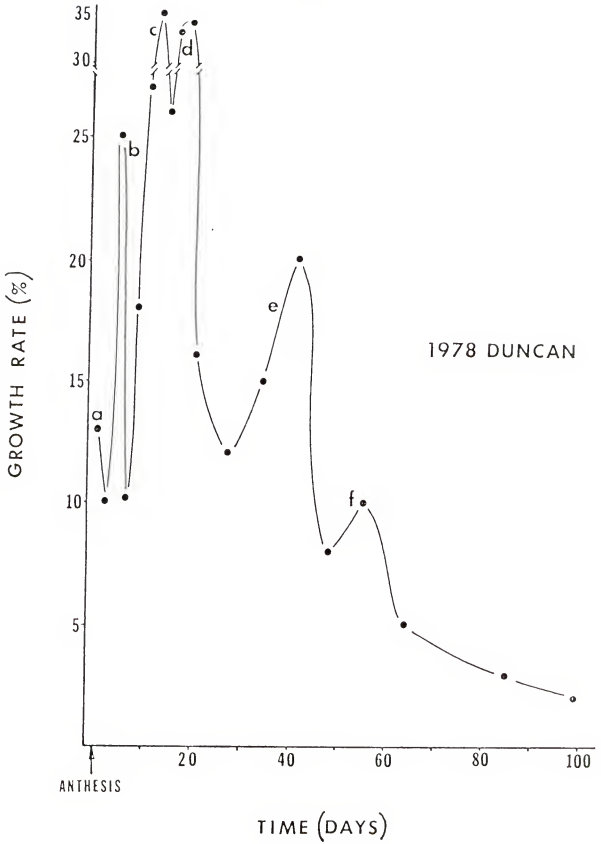

35
30
25
20
15
10
5
GROWTH RATE (%)
a
b
c
d
e
f
1978 DUNCAN
20
40
60
80
100
ANTHESIS
TIME (DAYS)

Figure 4. Mean daily percent growth rate of 'Marsh' grapefruit during the early fruit growth period, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

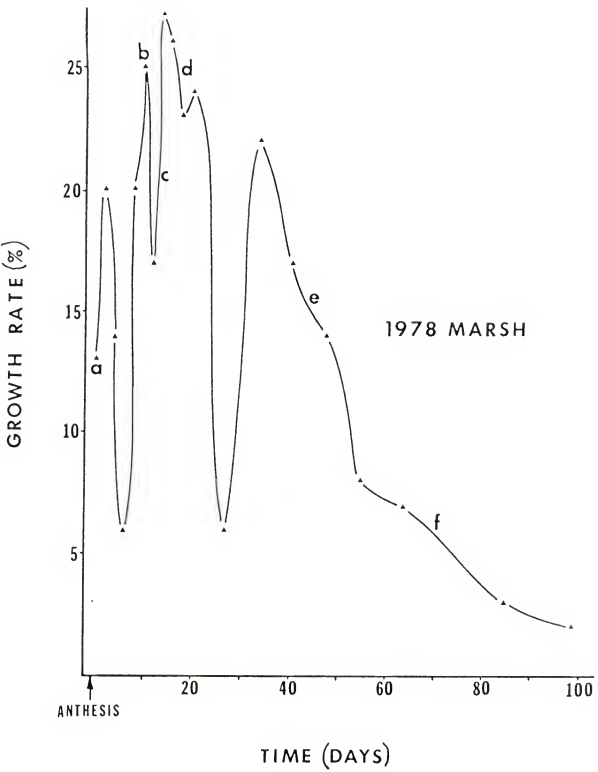

GROWTH RATE (%)
25
20
15
10
5
a
b
c
d
e
f
1978 MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 5. Mean daily percent growth rate of 'Duncan' grapefruit during the early fruit growth period, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

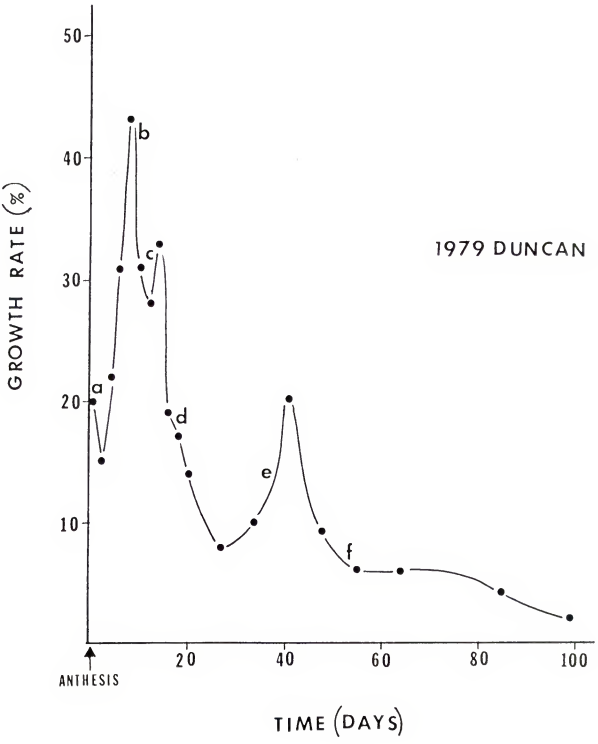

GROWTH RATE (%)
50
40
30
20
10
a
b
c
d
e
f
1979 DUNCAN
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 6. Mean daily percent growth rate of 'Marsh' grapefruit during the early fruit growth period, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

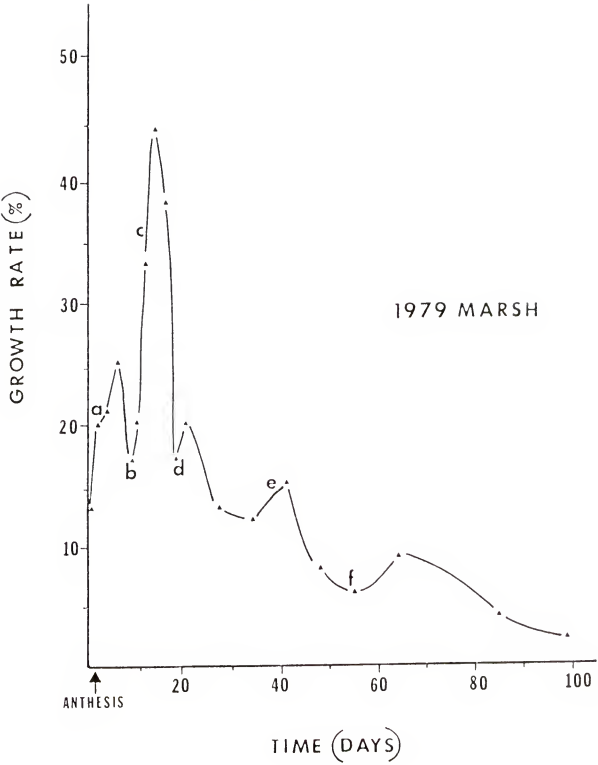

GROWTH RATE (%)
50
40
30
20
10
1979 MARSH
a
b
c
d
e
f
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Table 3. Young fruit drop of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1979.

Days after FB[z]	Fruit Drop			
	'Duncan'		'Marsh'	
	No. Fruit[y]	(%)[x]	No. Fruit[y]	(%)[x]
FB -2	729	--	827	--
FB	815	13	904	11
+2	894	11	979	13
+4	1011	16	1116	18
+6	1513	29	1428	25
PF +8	1419	24	1290	21
+10	1617	37	1507	32
+12	1322	20	1391	23
+14	1274	16	1253	14
+16	1105	12	1087	10
+18	924	12	904	8
+20	713	9	608	6
+27	387	6	312	4
+34	143	6	161	7
+41	102	5	94	4
+48	63	4	58	3
+55	112	11	91	10
+64	89	9	78	8

Table 3. (Continued)

Days after FB[z]	Fruit Drop			
	'Duncan'		'Marsh'	
	No. Fruit[y]	(%)[x]	No. Fruit[y]	(%)[x]
+85	21	4	18	3
+99	9	2	7	1

[z]Days before (-) or after (+) full bloom (FB); petal fall (PF).

[y]Counts of fruit drop onto saran cloth-covered frames placed under the tree. (Total of 6 trees.)

[x]Percent fruit drop =

$$\frac{\text{Number of fruit abscised between sampling dates}}{\text{Number of fruit at preceding sampling date}} \times 100;$$

calculated from branch counts including both bouquet and leafy bloom.

Fruit drop was high 2 days before approximately full bloom and remained high until petal fall and for an additional 2 weeks. A late peak of relatively high fruit drop occurred 47 to 77 days after petal fall.

Fruit falling in the first peak of drop has been attributed to nutritional, hormonal and other external factors (9, 11, 22, 51, 52, 75, 79, 88). Reports that citrus trees usually bloom heavily but only a comparatively small percentage of flowers set fruit, are well established (11, 24, 52).

The second peak of fruit drop occurred in late May and early June 1979 for both cultivars. Previous reports (11, 24, 75, 79, 88) have indicated environmental stresses, particularly temperature and drought as causes of fruit drop.

Pollen Tube Growth and Seed Development

The sexual process from pollen germination on the stigma through early embryo development were investigated in order to determine whether any of the various events in this process are related to levels of endogenous growth substances. A summary of these events is given in Table 4.

Table 4. Pollen tube growth and ovule development of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1978, 1979.

Days after FB[y]	'Duncan'[z]		'Marsh'[z]	
	1978	1979	1978	1979
FB	PT in stigmatic knob	PT in stigmatic knob	Rare PT in stigmatic knob (Sterile pollen grains)	Rare PT in stigmatic knob (Sterile pollen grains)
+(2-4)	PT in style	PT in style	Rare to none PT in style	Rare to none PT in style
+6	PT in style, none in ovary or ovule	PT in ovary, ovule; styles beginning to abscise	Ovule degeneration (contraction of integuments), occasional functional ovule	Ovule degeneration (contraction of integuments), occasional functional ovule
PF + 8	PT in ovary, ovule; styles beginning to abscise	PT in ovule	Continuous degeneration of ovules with occasional functional ones, styles abscised	Continuous degeneration of ovules with occasional functional ones, styles abscised
+10	PT in ovule	PT in ovule; styles abscised, division of endosperm	Continuous degeneration of ovules with occasional functional ones, styles abscised	Continuous degeneration of ovules with occasional functional ones, styles abscised
+12	PT in ovule, styles abscised, division of endosperm	Developing endosperm	Continuous degeneration of ovules with occasional functional ones, styles abscised	Continuous degeneration of ovules with occasional functional ones, styles abscised
+(14-34)	Free multinucleate endosperm	Free multinucleate endosperm	Continuous degeneration of ovules with occasional functional ones, styles abscised	Continuous degeneration of ovules with occasional functional ones, styles abscised
+(34-41)	Free multinucleate endosperm	Division of zygote		
+(41-48)	Division of zygote	Division of zygote, nucellar embryo formation		
+(55-65)	Division of zygote developing endosperm, nucellar embryo	Division of zygote, endosperm turned cellular		
+(65-99)	Developing embryo, cellular endosperm	Developing embryo, cellular endosperm		

[z]PT: pollen tubes

[y]Days after (+) full bloom (FB); petal fall(PF)

'Duncan' Grapefruit

Pollen grains of 'Duncan' germinated well on the stigmatic surface (Fig. 7) and pollen tubes grew through the style as indicated by the presence of callose plugs (Fig. 8). The resulting pollen tubes were seen in ovary and ovules 8 and 6 days after anthesis in 1978 and 1979, respectively (Fig. 9, 10). Initial division of endosperm (multinucleate endosperm) was observed 10 and 12 days following anthesis (Fig. 11) and it became cellular in the late stage of seed development (Fig. 12).

The zygote remained single celled mononucleated for several weeks (Fig. 13). The first evidence of zygotic division was observed 41 and 34 days after anthesis in 1978 and 1979, respectively. In both cases, a binucleated single cell zygote was observed (Fig. 14). The sexual embryo was attached to the embryo sac wall near the micropyle by a thin suspensor (Fig. 12). First signs of meristematic activity in nucellar cells were observed slightly after the endosperm nucleus had already divided (Fig. 14). Development of cells from the nucellus into the endosperm occurred 8 to 7 weeks after anthesis in 1978 and 1979, respectively.

Most previous research and this research failed to determine actual syngamy or fertilization, and only estimates have been made as to the time of fertilization (21, 83).

Figure 7. Pollen grain of 'Duncan' grapefruit on the stigmatic surface; pg, pollen grain; PT, pollen tube, sh, stigmatic hairs.

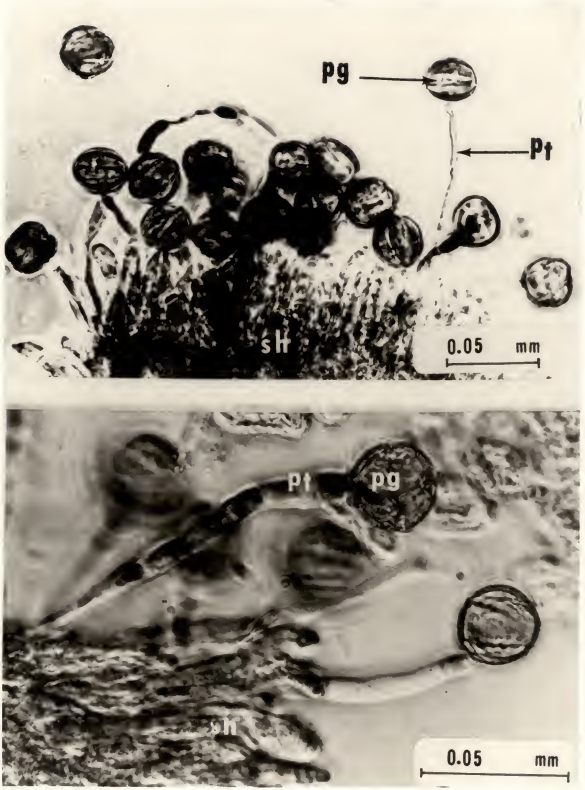

pg
pt
sh
0.05 mm
pt
pg
sh
0.05 mm

Figure 8. Callose plugs in pollen tubes in 'Duncan' grapefruit style; cp, callose plugs.

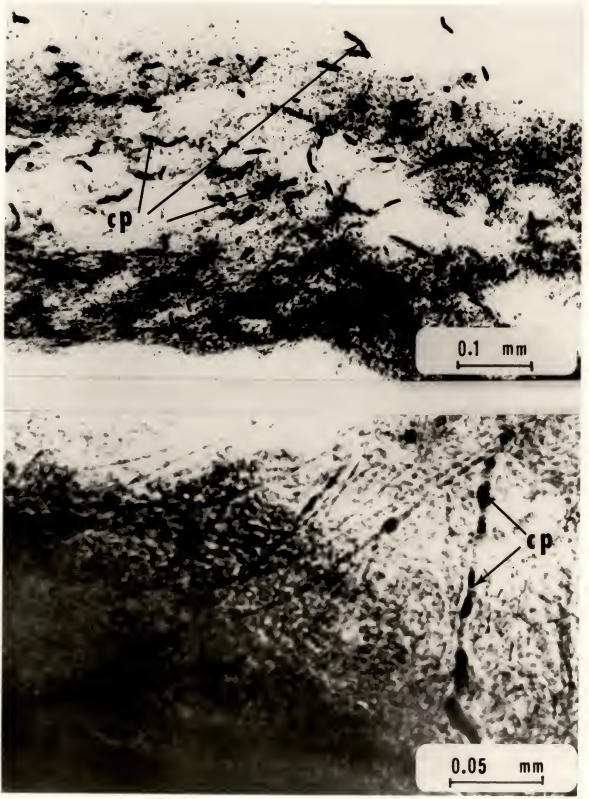
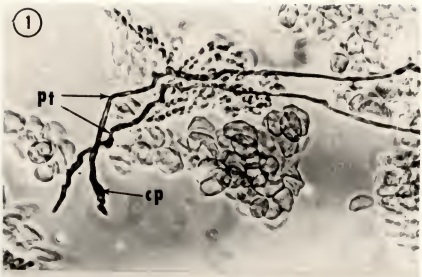
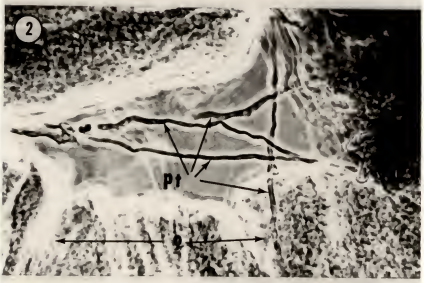

cp
0.1 mm
cp
0.05 mm

Figure 9. Pollen tubes in 'Duncan' ovary, 6 to 8 days after anthesis; cp, callose plugs; pt, pollen tube; o, ovule (1-2). Pollen tube (cp, callose plugs) approaching the micropyle (m) of an ovule (o); oi, outer integument; ii, inner integument (3).


1
Pt
cp


2
Pt

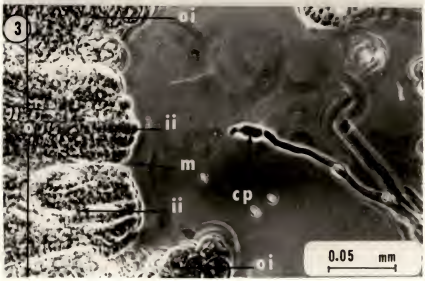

3
oi
ii
m
cp
ii
oi
0.05 mm

Figure 10. Pollen tubes in 'Duncan' ovules 6 to 8 days after anthesis; pt, pollen tube; m, micropyle; ii, inner integument; n, nucellus.

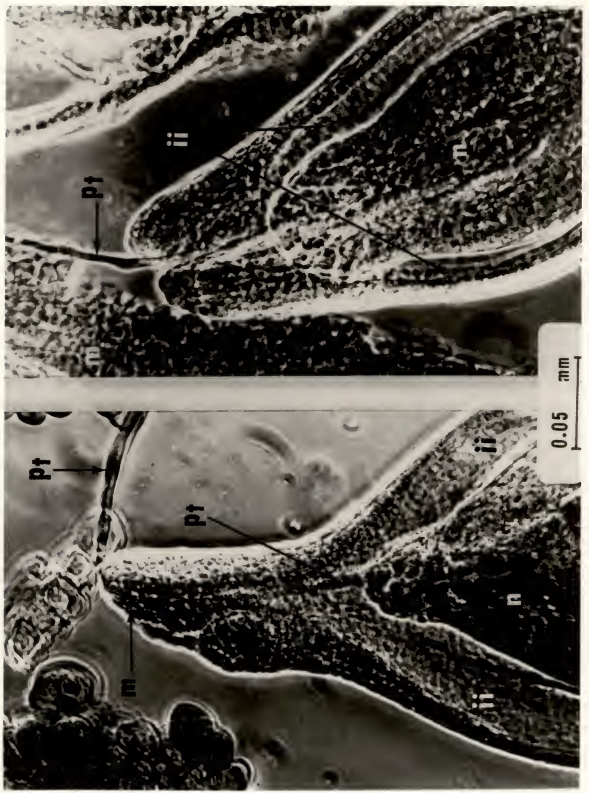

Pt
Pt
Pt
m
ii
ii
n
n
0.05 mm

Figure 11. Multinucleate endosperm in 'Duncan' ovule, 10 and 12 days after anthesis; ne, nuclear endosperm; es, embryo sac; n, nucellus.

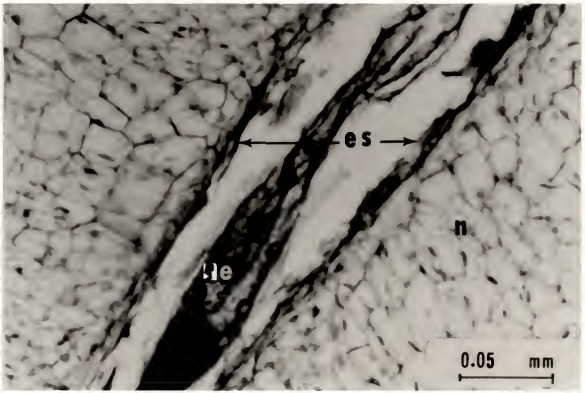
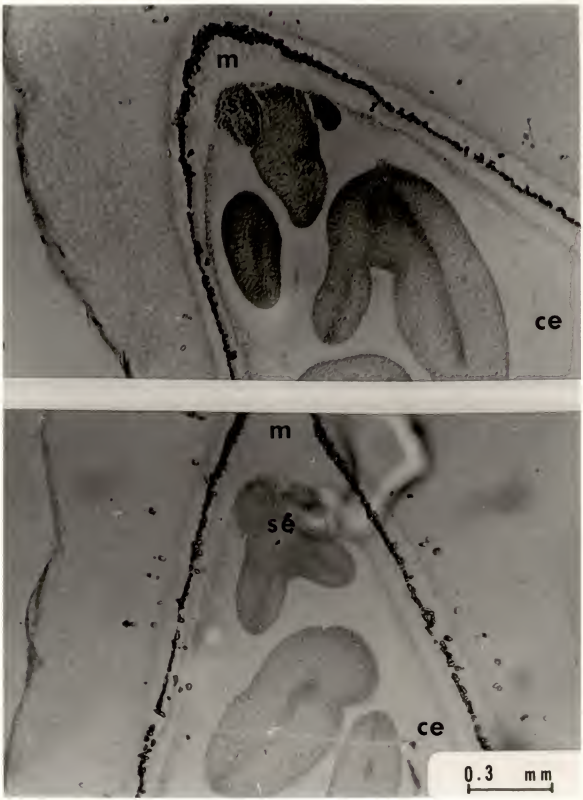

es
n
0.05 mm

Figure 12. Sexual embryo and cellular endosperm of 'Duncan' ovule in late stage of seed development; ce, cellular endosperm; m, micropyle; se, sexual embryo.


m
ce
m
sé
ce
0.3 mm

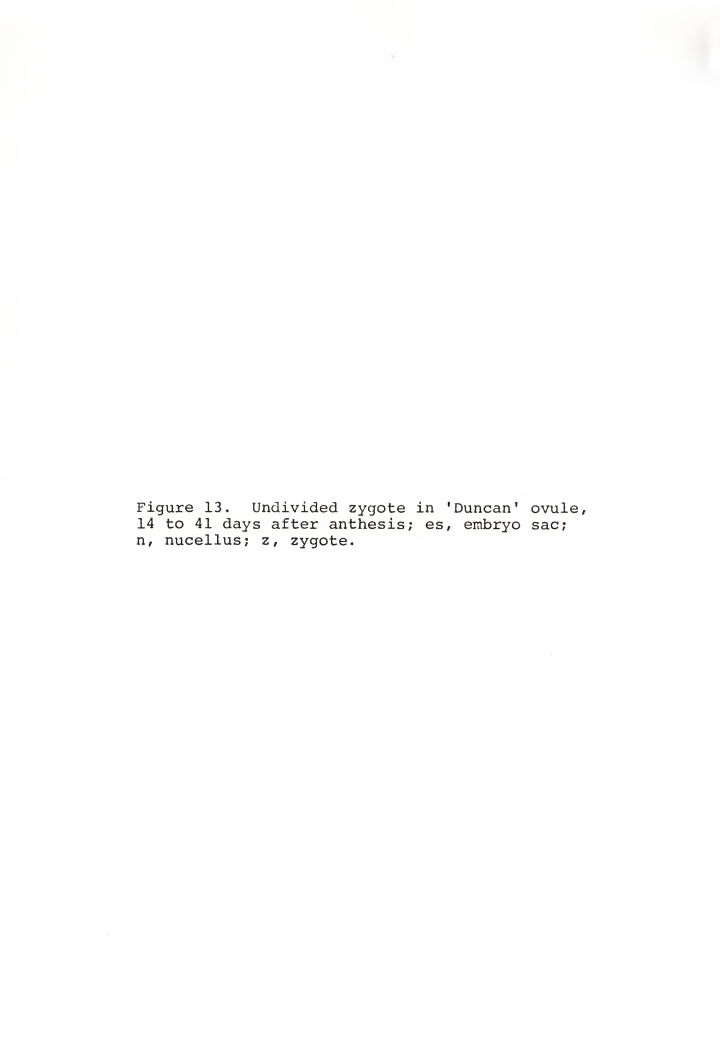


Figure 13. Undivided zygote in 'Duncan' ovule, 14 to 41 days after anthesis; es, embryo sac; n, nucellus; z, zygote.

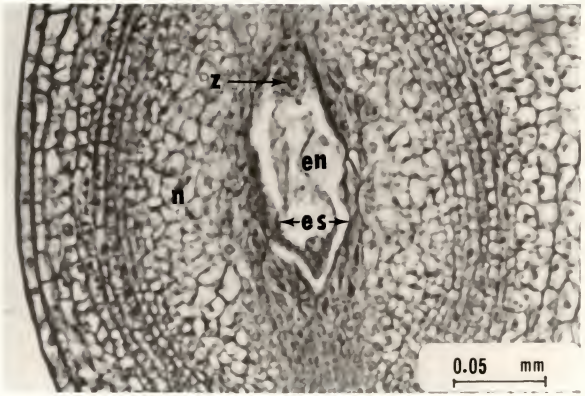
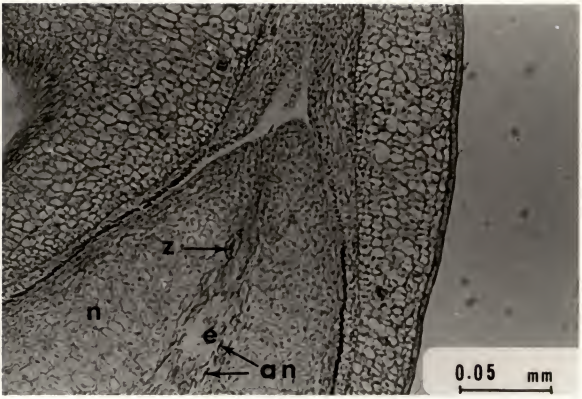

z
en
n
es
0.05 mm

Figure 14. Binucleated zygote and active nucellar cells, 34 and 41 days after anthesis; e, endosperm; n, nucellus; z, zygote; an, active nucellar cell.


z
n
é
an
0.05 mm

Pollen tubes were seen in the 'Duncan' ovules 8 and 6 days after anthesis and initial endosperm division was observed 12 and 10 days later in 1978 and 1979, respectively. Thus, it is reasonable to assume that fertilization took place between the time of entrance of the pollen tube in the ovule and the first signs of the endosperm division. Using this assumption, the data agree well with observations by Hensz (83) and Carlos (21). Conflicting results have been reported in satsuma and trifoliate orange (29). The latter is a different genus so the difference between it and 'Duncan' is not refutable; however, environmental stresses have been suggested as a cause of the short time (30 hours) to fertilization that has occurred in satsuma.

The occurrence of endosperm development is in approximate agreement with reports of Carlos (21), Bacchi (7) and Hensz (83). Endosperm nuclei were scattered throughout the embryo sac (Fig. 11) and appeared to be attached to a thin-layered cytoplasm. The cellular stage of endosperm was not observed in Hensz's (83) study, since his work ceased 40 days after pollination. Bacchi (7) also did not find cellular endosperm during the 67 days of his sampling period.

The developing zygote remained with no visible changes for 6 and 5 weeks after fertilization in 1978 and 1979, respectively. The length of the period in which the

zygote did not divide is similar to that previously reported for 'Orlando' tangelo (21, 83). Similar rest periods were noticed in 'Foster' grapefruit and trifoliate orange (7). Further development of the zygote and the sexual embryo up to termination of sampling, generally followed the same trend of development observed in some citrus cultivars (21).

Nucellar embryos reportedly develop after a zygotic embryo division (21). This was true in these studies as well.

'Marsh' Grapefruit

Microscopic examination of squashed 'Marsh' pistils showed no pollen tube germinating on the stigmatic surface (Fig. 15). Occasional callose plugs (Fig. 15), however, were observed in the styles in both seasons. Pollen tubes were not observed entering ovaries or ovules up to 16 days after anthesis, when microscopic observations were stopped. The ovules degenerated during this period and functional ovules were rarely observed by the 14th day after anthesis. Degenerate ovules were characterized by contraction of integuments from other ovule tissues (Fig. 16, 17).

Pollen and ovule sterility has been reported in 'Marsh' and 'Thompson' grapefruit (58, 90) as well as other citrus cultivars (155). Microscopic observations of 'Marsh' pollen and ovule in this study revealed similar results.

Figure 15. Ungerminated pollen grains of 'Marsh' grapefruit on the stigmatic surface and occasional callose plugs in the style; pg, pollen grains; ss, stigmatic surface; cp, callose plugs; vb, vascular bundle.

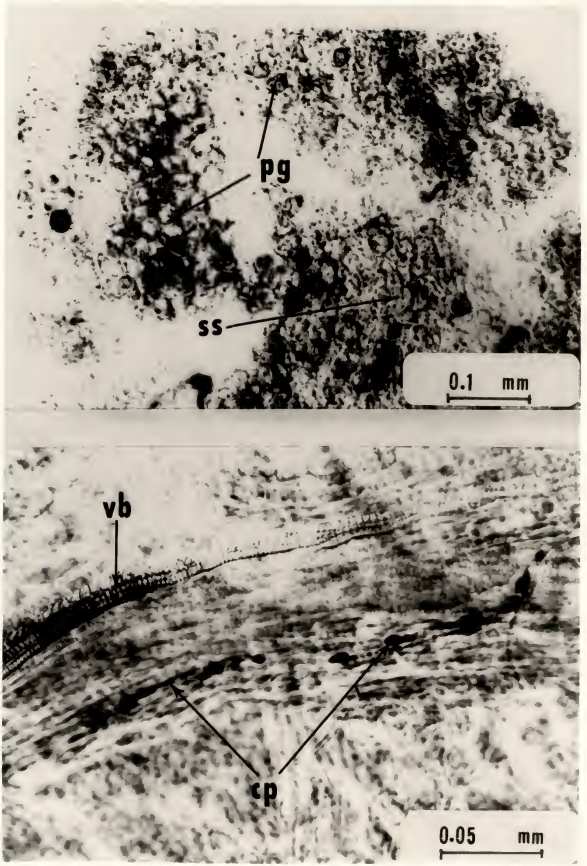

pg
ss
0.1 mm
vb
cp
0.05 mm

Figure 16. Early stage of nucellar contraction in 'Marsh' ovule 6 days after anthesis; ii, inner integument; oi, outer integument; m, micropyle; n, nucellus.

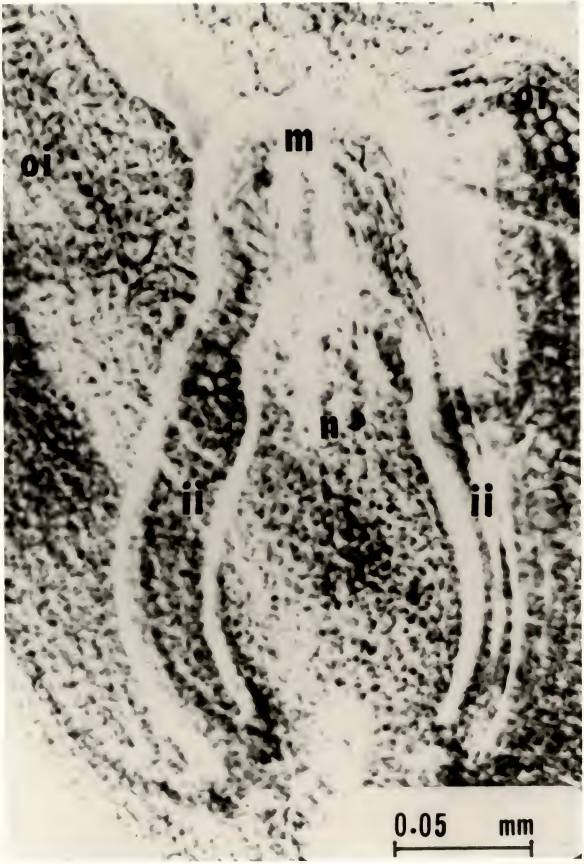
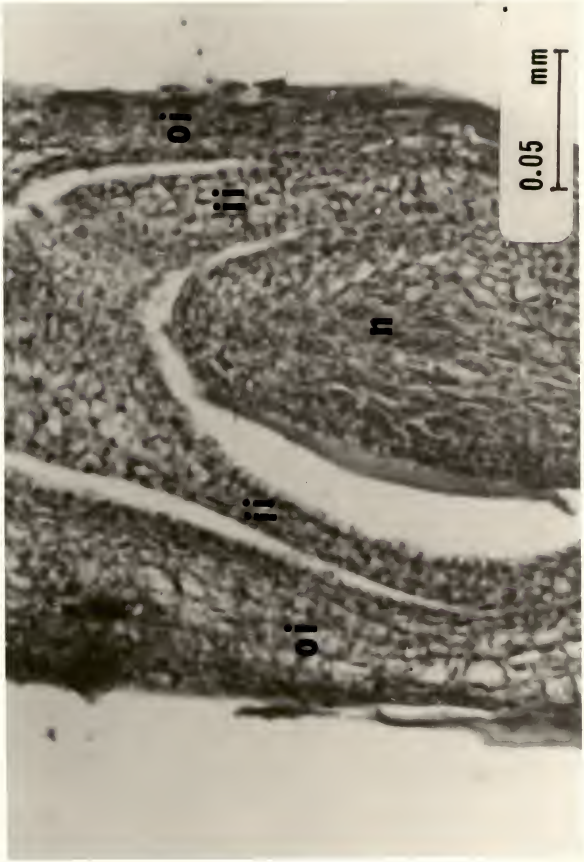

oi
m
oi
n
ii
ii
0.05 mm

Figure 17. Advanced stage of nucellar contraction in 'Marsh' ovule 14 days after anthesis; ii, inner integument; oi, outer integument; n, nucellus.


oi
ii
n
iii
oi
0.05 mm

Growth Substances

Verification of Analytical Procedure

Several methods have been utilized in previous years to investigate the nature of growth substances in plants (33, 49, 51, 61, 140, 203, 205). Difficulties have been encountered due to lack of adequate analytical techniques. Much improvement has been made in recent years, however, in development of chromatographic and spectroscopic procedures (18, 166, 203). Gas chromatography coupled with mass spectroscopy (GC-MS) is currently the best method for positive identification of growth substances in plants. This technique, however, is expensive and frequently requires large samples and extensive purification of extracts before analysis. Thus, a simpler much improved HPLC method was adapted (Fig. 1). The barley endosperm bioassay was used for GA determination since it was reported (190, 203) to be 100 to 1,000 times more sensitive than the fluorescence procedure. Even so, determinations of growth substances occasionally give unsatisfactory results. Accordingly, verification of the analytical procedure used in this research was necessary.

Amounts of IAA and ABA present in each sample were calculated by isotope dilution. Average recovery of IAA was 62% and ABA was 85% as measured by percentage recovery of added isotopes. The amounts of GA were

calculated based on an average recovery of 71% measured by addition of a GA_3 standard to samples prior to subjecting to the isolation procedure.

Reproducibility obtained with this system by running duplicates of 3 different 10 g samples is shown in Table 5. The HPLC system used in this research presented good separation of IAA and ABA (Fig. 18). GA_1 and GA_3 were eluted in the same region as IAA but did not interfere due to different analytical procedures.

IAA and ABA identifications in the present study were based on data of previous reports in this laboratory. The characteristic indolo-α-pyrone fluorescent method has been used for IAA verification and ABA has been confirmed by its isomerization to trans-trans/ABA when exposed to UV and measured in the HPLC analytical system (49, 203).

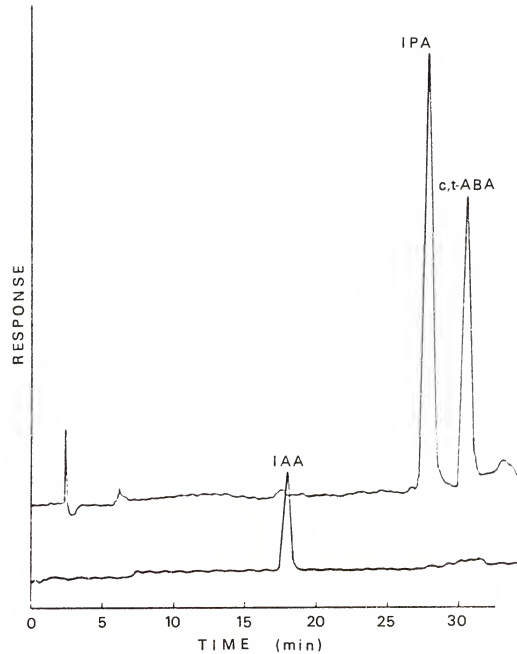
No attempt was made to separate GA_1 and GA_3 in this study and all GA results are calculated as GA_3 equivalents in the barley bioassay system. GA_4 and GA_7 are well separated from dihydroxy gibberellins in this HPLC system. Detectable levels of GA_4 and GA_7, however, were not found.

Reports on quantitative and qualitative levels of endogenous growth substances in general are very limited and there are none for 'Duncan' and 'Marsh' grapefruit cultivars. Only a few studies provide information on

Table 5. IAA, GA and ABA reproducibility in duplicates of 3 different samples using the HPLC system.

	IAA		GA		ABA	
	A	B	A	B	A	B
Sample	ng/g fresh weight					
1	352	363	0.31	0.28	129	140
2	78	64	0.14	0.17	28	19
3	418	426	0.37	0.31	114	129

Figure 18. Chromatographic separation of IAA and ABA using the HPLC system; IPA, Indolepropionic acid.


IPA
c,t-ABA
IAA
RESPONSE
0
5
10
15
20
25
30
TIME (min)

concentration of growth substances occurring in plant tissues (25, 51, 53, 61, 63, 65, 72, 81, 83, 203) and a lesser number include careful identification of the compound studied (51, 63, 65, 140, 142, 203). Even with the increased precision of recently developed methods, reports on levels of growth substances in plant tissue vary considerably. Ranges of 75 to 690 ng IAA and 125 to 180 ng ABA per gram fresh weight have been reported in young developing fruit of satsuma mandarin (183). Concentrations of 60.8-150.5 ng GA per gram fresh weight have also been reported in young navel orange fruits (205).

Levels of 21-766 ng IAA, 13-395 ng ABA and 0.062-0.435 ng GA per gram fresh weight were obtained in this study. Considerable variation in amounts of these growth substances has been reported (183, 205), but there is reason to believe that the data are accurate. Both radioactive IAA and ABA standards were added to every sample analyzed and the consistency of results strengthened confidence in these data. In addition, GA was consistently eluted in the same fraction from the preparative HPLC system and its activity was always detected in the bioassay with the automated analyzer in all samples. Recovery of GA_3 standard when added to the sample for verification of the procedure also indicated that inhibitors and other compounds were not important factors in GA determination.

Growth Substance Changes During Young Fruit Development

Auxins

Free IAA content was low at anthesis and increased sharply to a peak 11 and 14 days after anthesis in 1978 and 1979, respectively (Fig. 19, 20). Seedless 'Marsh' fruit was slightly higher in IAA content than 'Duncan' and maintained this difference for approximately 20-22 days after anthesis. 'Duncan' fruit, however, developed slightly higher IAA levels than 'Marsh' as the season progressed.

Total amounts of IAA, calculated on a per fruit basis for both cultivars (Fig. 21, 22), remained low during the first 10 days after anthesis, but increased greatly thereafter in both seasons.

Highest levels of free IAA were observed during the early stage of fruit development. This pattern is in approximate agreement with reports on seasonal changes of IAA in fruit of some other citrus cultivars (183) and other crops (126). The likelihood IAA is involved in events that trigger fruit set has been reported (72). This assumption may be held for both cultivars in this study. Others (81, 118), on the other hand, have reported more than 1 peak of IAA during the early period of fruit development and another late in the season.

Figure 19. IAA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

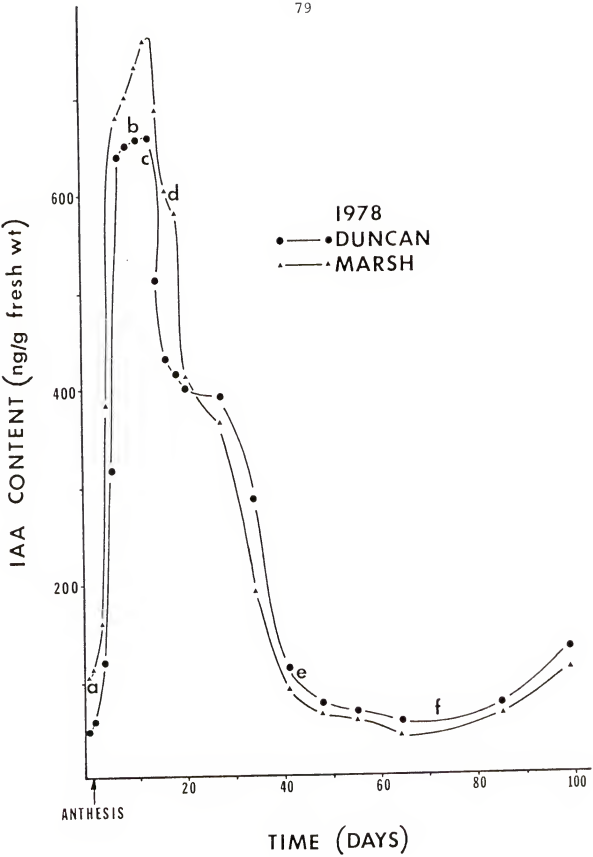

IAA CONTENT (ng/g fresh wt)
600
400
200
1978
DUNCAN
MARSH
a
b
c
d
e
f
20
40
60
80
100
ANTHESIS
TIME (DAYS)

Figure 20. IAA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zyogte; f, Cellular endosperm.

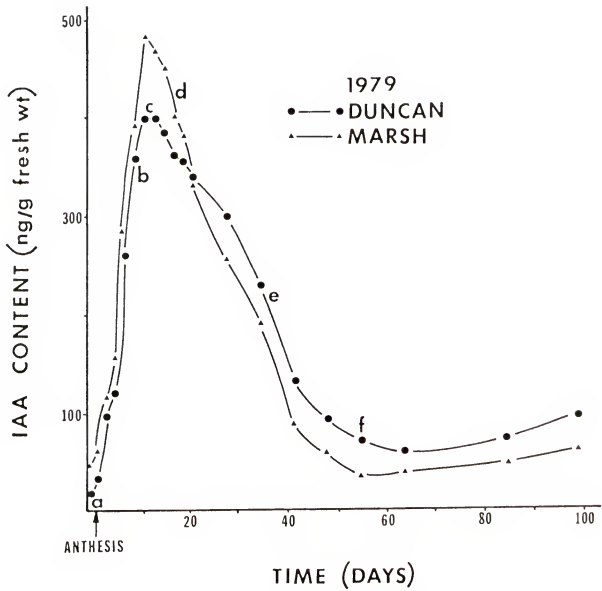

500
300
100
IAA CONTENT (ng/g fresh wt)
1979
DUNCAN
MARSH
a
b
c
d
e
f
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 21. Total amount of IAA per fruit of 'Duncan' and 'Marsh' grapefruit during the early period of fruit growth, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

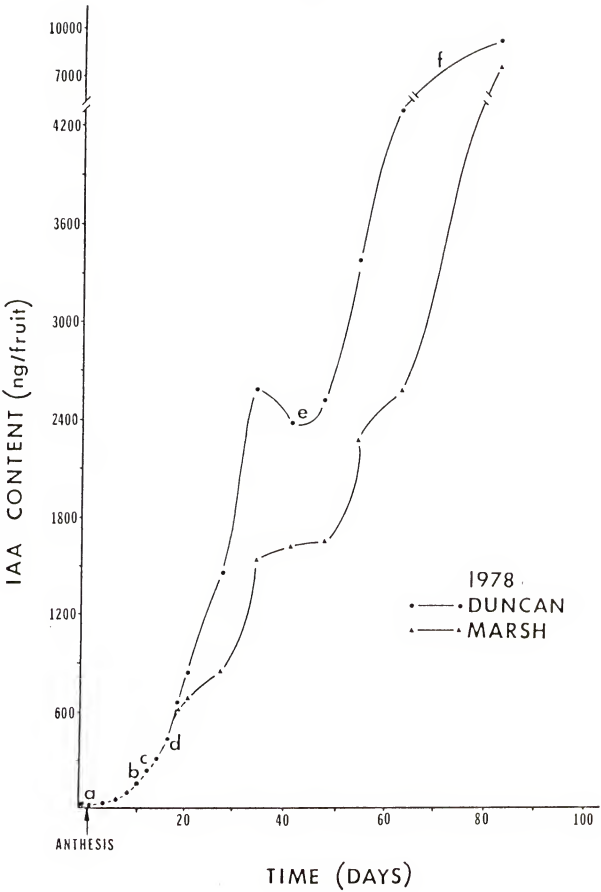
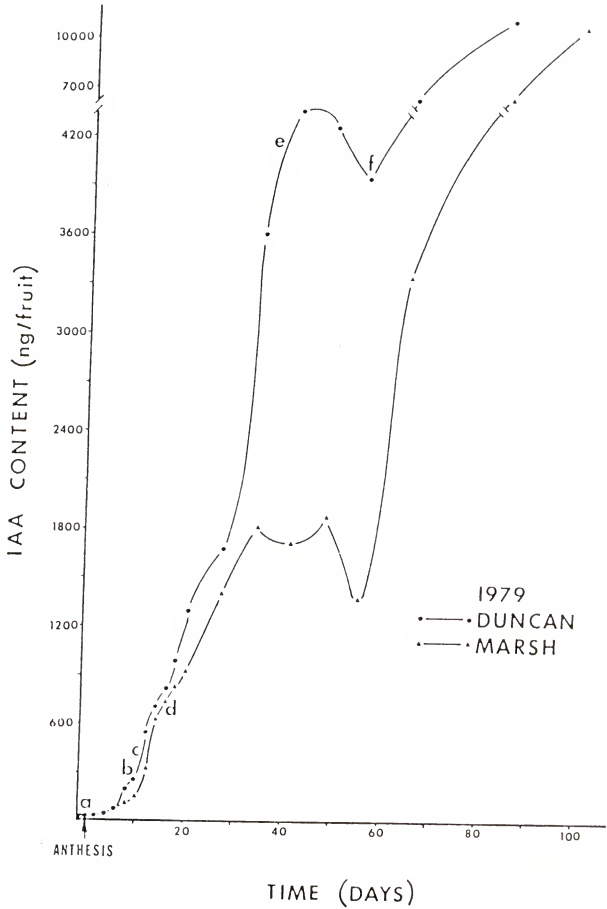

10000
7000
4200
3600
3000
2400
1800
1200
600
IAA CONTENT (ng/fruit)
a
b
c
d
e
f
1978
DUNCAN
MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 22. Total amount of IAA per fruit of 'Duncan' and 'Marsh' grapegruit during the early period of fruit growth, 1979. a, Pollination; b, Fertilization, c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


IAA CONTENT (ng/fruit)
10000
7000
4200
3600
3000
2400
1800
1200
600
a
b
c
d
e
f
1979
DUNCAN
MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Only 1 peak was observed in each season for each cultivar in this study. This is not surprising, because growth substances are influenced by the status of the tree, environmental conditions and other factors (87, 119, 129, 149, 158).

Gibberellins

Patterns of change in GA content were quite characteristic in both seasons (Fig. 23, 24). GA levels in 1978 were high at anthesis, dropped to a slightly lower level 2 days after anthesis and increased within 10 days to relatively the same level found at anthesis. Another peak was observed 27 days after anthesis. GA content in 1979 was low at anthesis and followed by 2 pronounced peaks 8 and 27 days later.

Total GA on a per fruit basis followed a similar pattern to that described for IAA (Fig. 25, 26).

Seasonal changes in endogenous amounts of GA in young fruit of both 'Duncan' and 'Marsh' followed a pattern similar to those reported for fruit of other citrus cultivars (205). Levels of GA, however, were high just before anthesis in 1978, compared to low levels at the same period in 1979. This trend was possibly due to tree condition as well as environmental factors at the time of sampling.

Figure 23. GA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

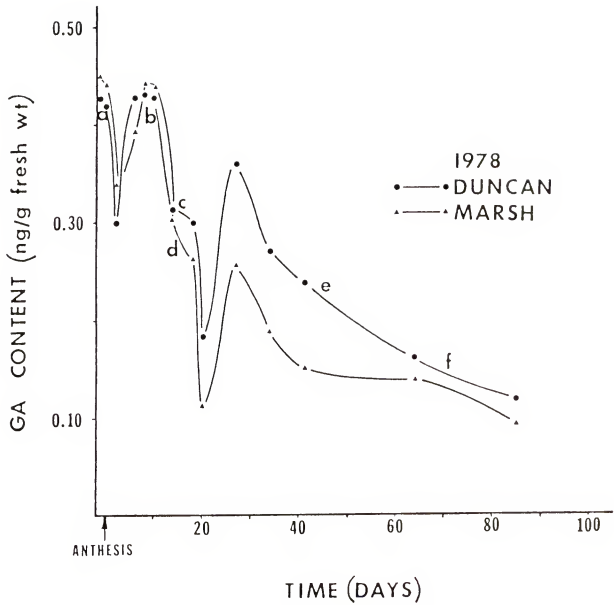

0.50
0.30
0.10
GA CONTENT (ng/g fresh wt)
a
b
c
d
e
f
1978
DUNCAN
MARSH
20
40
60
80
100
ANTHESIS
TIME (DAYS)

Figure 24. GA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

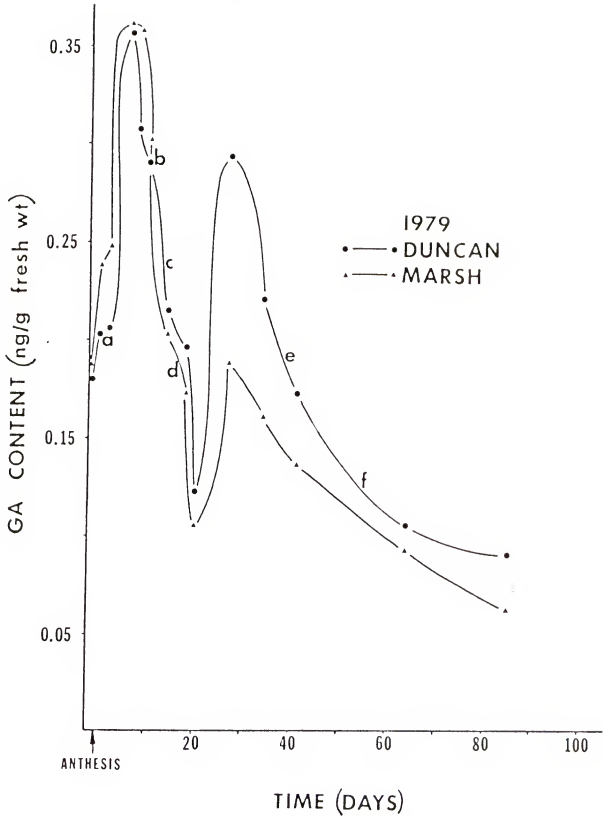

0.35
0.25
0.15
0.05
GA CONTENT (ng/g fresh wt)
1979
DUNCAN
MARSH
a
b
c
d
e
f
20
40
60
80
100
ANTHESIS
TIME (DAYS)

Figure 25. Total amount of GA per fruit of 'Duncan' and 'Marsh' grapefruit during the early period of fruit growth, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

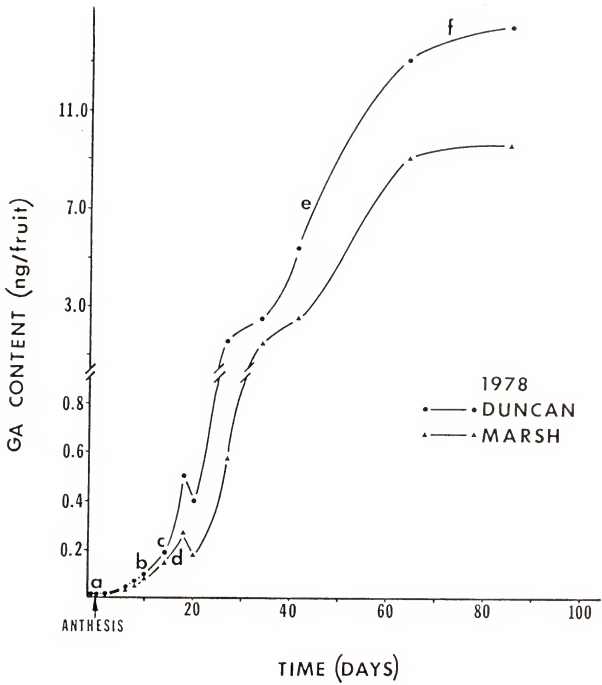
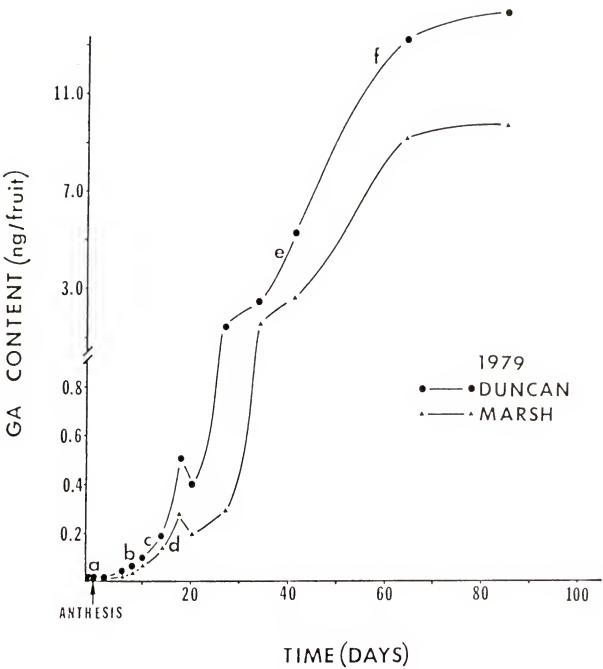

GA CONTENT (ng/fruit)
11.0
7.0
3.0
0.8
0.6
0.4
0.2
a
b
c
d
e
f
ANTHESIS
20
40
60
80
100
TIME (DAYS)
1978
DUNCAN
MARSH

Figure 26. Total amount of GA per fruit of 'Duncan' and 'Marsh' grapefruit during the early period of fruit growth, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


GA CONTENT (ng/fruit)
11.0
7.0
3.0
0.8
0.6
0.4
0.2
a
b
c
d
e
f
20
40
60
80
100
ANTHESIS
TIME (DAYS)
1979
DUNCAN
MARSH

Abscisic Acid

In 1978, free ABA levels for both cultivars were characterized by 2 definite peaks that occurred at 12 and 50 days after anthesis, respectively (Fig. 27). ABA content in 1979 was low at anthesis, reached a peak 10 days later and declined sharply to a minimum level 65 days after anthesis (Fig. 28).

Total amounts of ABA per fruit (Fig. 29, 30) also followed a pattern similar to those described for IAA and GA. A small decrease in ABA level was observed late in the season for both cultivars.

Levels of ABA for both cultivars in 1979 followed relatively similar patterns reportcd for satsuma mandarin (183) in Japan. In 1978, 2 peaks of ABA were observed. Previous research indicated ABA and other environmental factors, particularly moisture, were closely related (87). Climatic data were not collected during this study but it is likely that such fluctuations in ABA levels in 1978 were partially moisture related.

Growth Substance Changes in Relation to Sexual Process

A major objective of this research was to detect any sequential changes in IAA, GA and ABA content in the developing young fruit of seedy 'Duncan' and seedless

Figure 27. ABA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

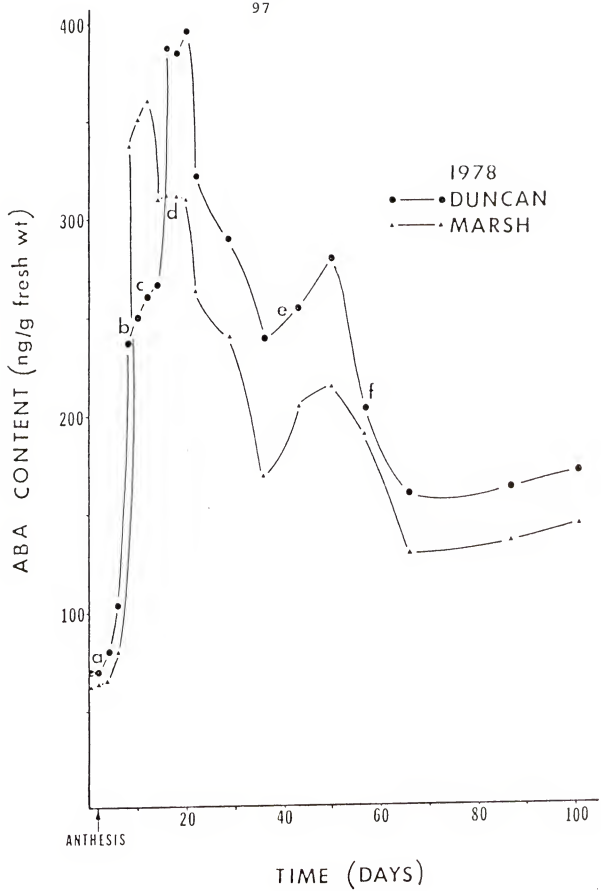

400
300
200
100
ABA CONTENT (ng/g fresh wt)
1978
DUNCAN
MARSH
a
b
c
d
e
f
20
40
60
80
100
ANTHESIS
TIME (DAYS)

Figure 28. ABA content of 'Duncan' and 'Marsh' grapefruit during the early fruit growth period, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

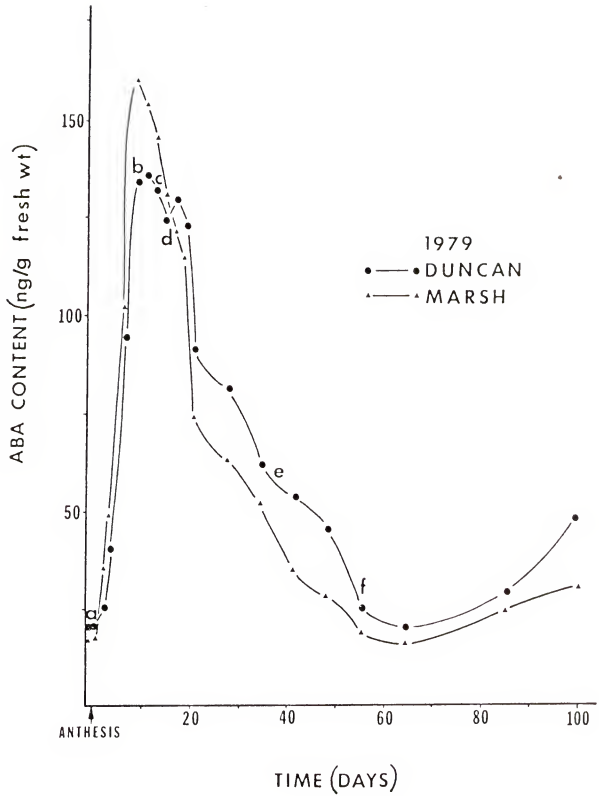

ABA CONTENT (ng/g fresh wt)
150
100
50
1979
DUNCAN
MARSH
a
b
c
d
e
f
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 29. Total amount of ABA per fruit of 'Duncan' and 'Marsh' grapefruit during the early period of fruit growth, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.

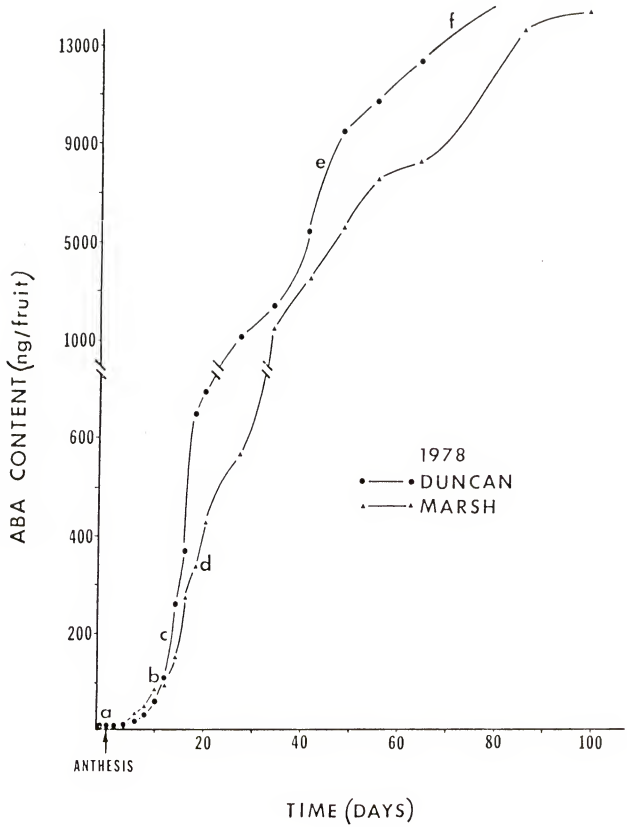
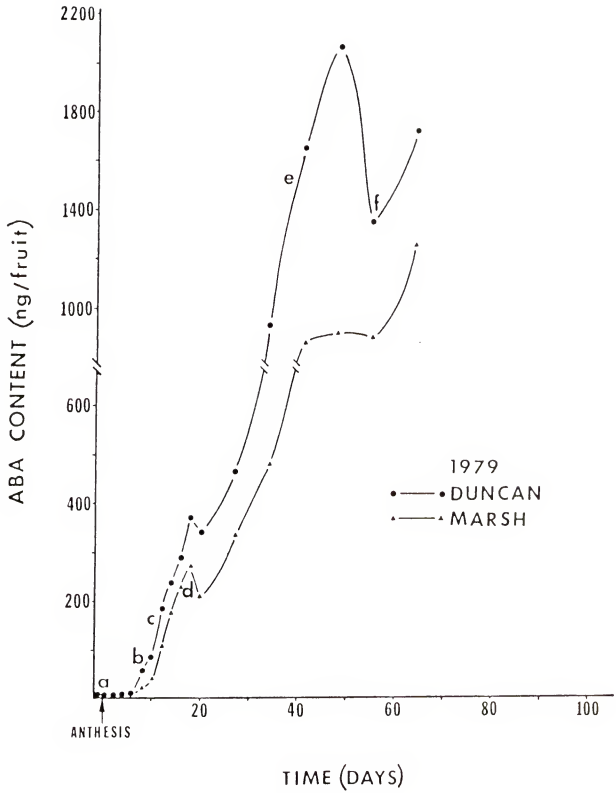

13000
9000
5000
1000
600
400
200
ABA CONTENT (ng/fruit)
a
b
c
d
e
f
1978
DUNCAN
MARSH
ANTHESIS
20
40
60
80
100
TIME (DAYS)

Figure 30. Total amount of ABA per fruit of 'Duncan' and 'Marsh' grapefruit during the early period of fruit growth, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


ABA CONTENT (ng/fruit)
2200
1800
1400
1000
600
400
200
a
b
c
d
e
f
ANTHESIS
20
40
60
80
100
TIME (DAYS)
1979
DUNCAN
MARSH

'Marsh' and to relate, if possible, such changes to the sexual process.

The schedule of the sexual process for both cultivars in both seasons was only slightly different.

Relation of Pollen Tube Growth to IAA, GA and ABA in Developing Young Fruit

Pollen tubes of 'Duncan' entered the ovary and ovules 8 and 6 days in 1978 and 1979, respectively (Fig. 15). No pollen tubes were observed in 'Marsh' ovules, since its pollen is almost entirely sterile (58, 90). Fertilization had not occurred by this time.

Levels of IAA and ABA in ovary of both cultivars in both seasons rose sharply after anthesis and reached high peaks just a few days after pollen tubes were observed in ovary and ovule of 'Duncan' (Fig. 9, 10, 19, 20, 27, 28). GA levels in 1979 (Fig. 24) followed a pattern similar to those of IAA and ABA. On the other hand, GA levels in 1978 (Fig. 25) were high at anthesis, dropped slightly and then increased shortly afterwards to a peak relatively equal to that at anthesis.

Levels of IAA, GA and ABA followed patterns similar to those described for seedy 'Duncan' despite the fact 'Marsh' is a parthenocarpic cultivar with sterile pollen and ovules. Levels of these growth substances, however, were slightly higher in 'Marsh' than 'Duncan'

during the early period of fruit growth. These differences were small in both seasons.

All 3 of the growth substances reached high levels in 'Duncan' ovaries after entrance of pollen tubes into the ovary and ovules as compared to those occurring when pollen tubes were in the style. This sequence of events indicated high levels of IAA, GA and ABA in the ovary were possibly associated with the presence of pollen tubes. The same hormonal trend was noticed in seedless 'Marsh.' One can speculate the existence of few functional ovules for several days after anthesis might have been responsible for high hormonal levels at this stage in 'Marsh.' Also, it is possible non-reproductive tissues in the fruit and even other tree parts particularly the leaves might be responsible for levels of hormones in parthenocarpic 'Marsh.' Previous work (72) suggested parthenocarpic fruit set was due to higher auxin content produced in the developing ovary and GA might affect fruit set by increasing IAA content (97, 111, 157, 159, 172, 173). Others (144, 145, 150, 191) reported the requirement of pollination stimulus to increase hormonal content in ovaries of seedy cultivars for proper fruit set. Hensz (83) reported an increase in promoter as well as inhibitor content of cross-pollinated 'Orlando' tangelo ovaries after they were penetrated by pollen tubes.

GA content in fruit of both cultivars dropped after anthesis in 1978. This observation is in disagreement with reports (83, 144, 145, 150, 172, 191, 205) pollination increases growth substances in ovaries of seedy cultivars. This hormonal drop was not noticed in the 1979 season, in which the GA pattern found, resembles those of previous reports (25, 102, 205).

Relation of Fertilization and Endosperm to IAA, GA and ABA in Young Developing Fruit

The actual fertilization process in 'Duncan' was not observed in either season. The time of occurrence can be estimated closely, however, as between the entrance of pollen tubes in ovules (Fig. 10) and initial evidence of endosperm division (Fig. 11). A time lapse of 4 days was noticed between the 2 events in both seasons. Levels of IAA, GA and ABA in fruit of both cultivars was relatively high during this period; however, they declined shortly afterwards (Fig. 19, 20, 23, 24, 27, 28). GA in both seasons, and ABA in 1978, rose to a second peak 17 days and 33 days after fertilization, respectively.

That IAA content remained high during this process is not uncommon but the rapid decline shortly afterwards is in contrast to previous reports (72, 144, 145), which have indicated an influx of endogenous IAA following fertilization. This evidence may have cast some

doubt on the relation between IAA and the sexual process. Previous research (89, 106, 110) showed application of auxin during the early period of fruit development did not affect fruit set in citrus. Thus, it may be inferred IAA is not involved in this critical period of fruit set. On the other hand, GA showed a second peak approximately 2 weeks after fertilization and initial division of endosperm. 'Marsh' fruit showed the same pattern with a slight difference in GA content. Thus, it is reasonable to assume this GA increase coincides with fertilization and endosperm division in seedy 'Duncan,' while the same pattern in 'Marsh' may be related to other fruit tissues and other occasional viable ovules. The former assumption was substantiated by reports fertilization (97, 111, 159, 172) and endosperm development (40, 124, 126, 146) increased growth substances in developing fruit. Changes in ABA content in both 'Duncan' and 'Marsh' fruit may be attributed to similar assumptions made for changes in IAA and GA in both cultivars in this section.

Relation of Other Sexual Events to IAA, GA and ABA in Young Developing Fruit

Zygotic division, cellular endosperm and nucellar embryo development were observed in 'Duncan' fruit relatively late in both seasons (Table 4). There was no indication of any relationship among these events and

levels of IAA, GA or ABA (Fig. 19, 20, 23, 24, 27, 28). Levels of these growth substances with the exception of ABA content in 1978, continued to decline during these sexual events. Growth substance content in 'Marsh' fruit showed the same trend; however, levels were slightly lower than those of 'Duncan.'

Results in this stage of fruit enlargement indicate that neither zygotic division nor subsequent sexual events were related to IAA, GA and ABA changes. It is very likely also, since seedless 'Marsh' followed a similar hormonal trend, fruit set at this stage depends upon a complex of internal and external factors in addition to the variables described in this study. Research in this stage of fruit development is limited, however, to a few reports which have related fruit growth to growth substances rather than the sexual events (81, 205).

Relation of Sexual Process and Growth Substances to Fruit Set

There was a very slight difference in content of growth substances between the 2 cultivars. Furthermore, changes in IAA, GA and ABA followed similar patterns in both cultivars (Fig. 19, 20, 23, 24, 27, 28) despite the fact 'Marsh' had sterile pollen and ovules and did not require the sexual process to set fruit. Certain events of the sexual process, namely pollination, was apparently

related to an increase in IAA, GA and ABA in ovaries of 'Duncan.' An exception to this was GA in the 1978 season (Fig. 25) in which it decreased but then increased to a relatively high level. These observations are consistent with previous reports (72, 100, 101, 144, 150).

There appears to be a limited relationship when the events characteristic of the sexual process are compared to the changes in levels of the growth substances studied. This conflicts with the concept that endogenous growth substances are produced as a result of the sexual process and are in turn responsible for fruit set and subsequent growth. The failure to find such an overall relationship could be due to limitations in methodology. It seems more likely, however, that the problem lies in the tissues analyzed. Entire ovaries were used and it is possible only certain tissues in the ovary are involved. If this is true, it is possible use of entire ovaries could have diluted levels of growth substances and resulted in misleading or erratic results. Another problem lies in the inability to select small fruits that will definitely set and produce fruit. Early in the fruit-setting period, healthy-appearing, green fruit will suddenly turn yellow and abscise. It is possible some of the healthy, green fruit sampled would have turned yellow soon after sampling and abscised. Such fruit might have already

had changes in levels of growth substances predisposing them to abscission at the time of sampling

Relation of Endogenous Amounts of IAA, GA and ABA to Fruit Growth

Growth rate of both 'Duncan' and 'Marsh' fruit, expressed as percentage of daily change in fruit fresh weight (Fig. 3, 4, 5, 6) showed the periodicity in growth exhibited by citrus fruits used in this study. These results are consistent with reports (8, 11, 14, 81, 205) of other citrus cultivars. Evidence that IAA and GA content regulate both cell division and cell enlargement has been reported (12, 16, 19, 76, 86, 118, 121, 170, 205).

Percentage growth rate of fruit of both cultivars in both seasons changed similarly to changes in IAA and GA levels (Fig. 19, 20, 23, 24) in this research. IAA relationship, however, continued only to 3-4 days after anthesis, while GA consistently increased before each growth response throughout the period of sampling. The intervals of delay between change in hormonal levels and growth responses ranged from 1 to 10 days for both IAA and GA. A definite relation was not observed between changes in ABA levels and fruit growth responses for both cultivars.

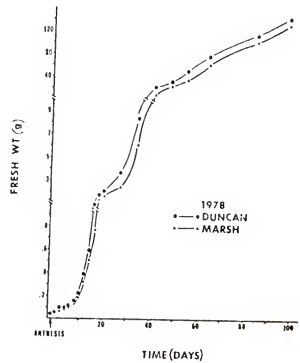
The IAA relationship was confined to Stage 1 of fruit growth while GA apparently was involved in Stage 1 as well as Stage 2. These results are similar to previous

reports (12, 16, 19, 81, 118, 126, 205) which indicated the involvement of IAA in Stage 1 when fruit growth was primarily due to cell division, and GA in Stage 1 as well as Stage 2 when fruit growth was predominantly due to cell enlargement

The results also show great increases in total IAA and GA content per fruit just before appreciable increases in fruit growth (Fig. 31, 32, 33, 34). These observations possibly indicate a relationship between these growth substances and fruit growth. Climatic data were not collected during this study; however, previous research (11) has indicated their relation to fruit growth responses, thus further investigation in this respect may be needed.

These relationships during this period of fruit growth in the present study may possibly be critical to responses of citrus trees to IAA or GA application to promote fruit set. Application of these growth substances when levels are low may promote fruit set, while undesirable results may be obtained when endogenous levels are high. Variable results have been obtained in previous work (26, 37, 41, 89, 106, 109, 129, 130) due to application of IAA and GA to individual citrus flowers or whole trees.

Figure 31. Mean fresh weight of young fruit of 'Duncan' and 'Marsh' grapefruit and seasonal changes in total IAA per fruit, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zyogte; f, Cellular endosperm.


FRESH WT (g)
1978
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)

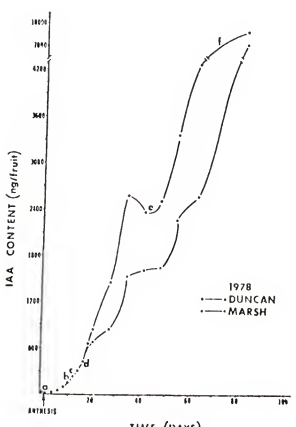
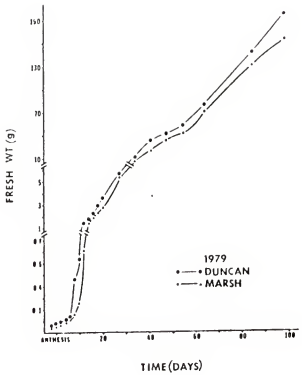

IAA CONTENT (ng/fruit)
1978
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)

Figure 32. Mean fresh weight of young fruit of 'Duncan' and 'Marsh grapefruit and seasonal changes in total IAA per fruit, 1979. a, Pollination; b, Fertilization, c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


FRESH WT (g)
1979
DUNCAN
MARSH
ANTHESIS
TIME(DAYS)

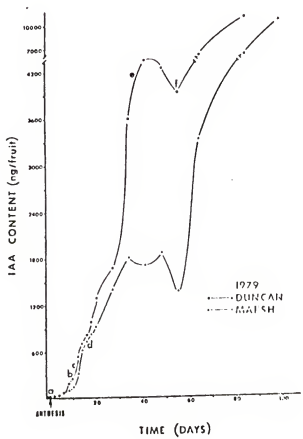
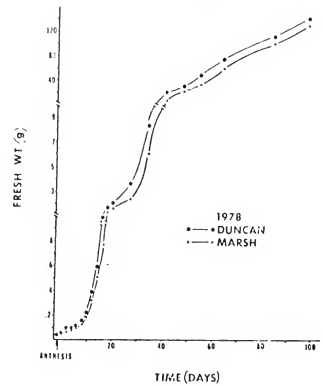
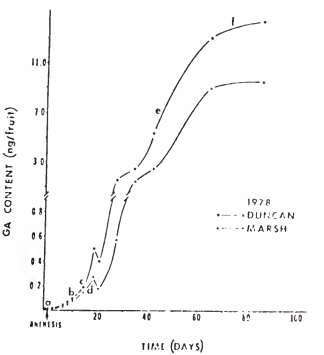

IAA CONTENT (ng/fruit)
1979
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)

Figure 33. Mean fresh weight of young fruit of 'Duncan' and 'Marsh' grapefruit and seasonal changes in total GA per fruit, 1978. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


FRESH WT (g)
1978
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)


GA CONTENT (ng/fruit)
1978
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)

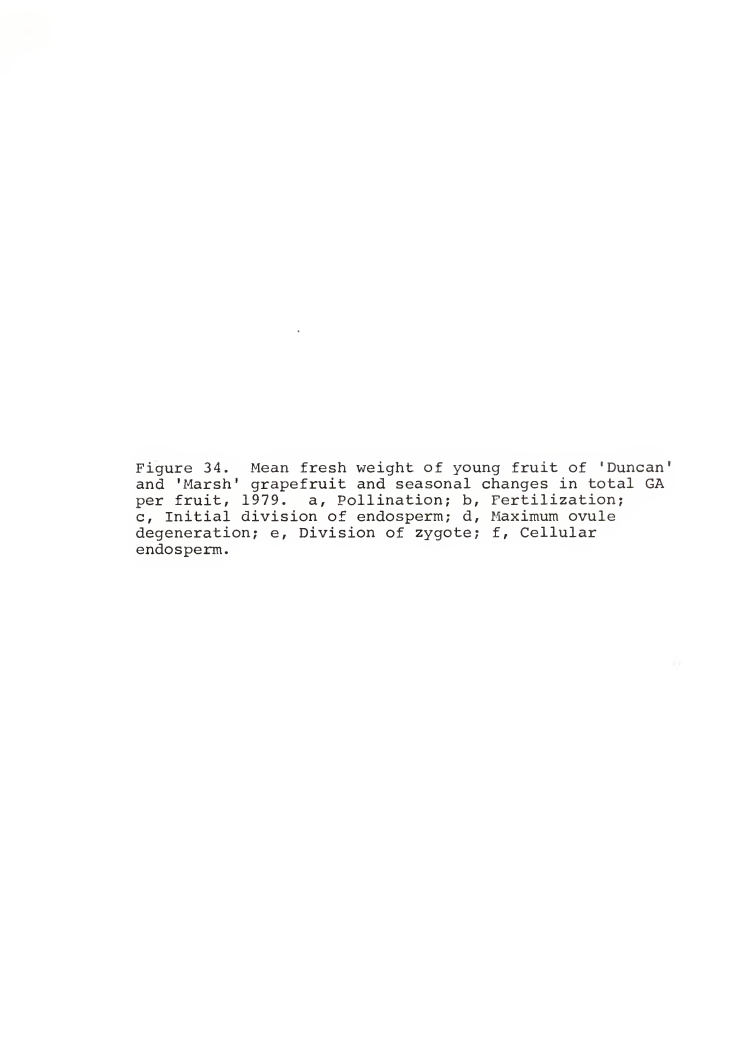
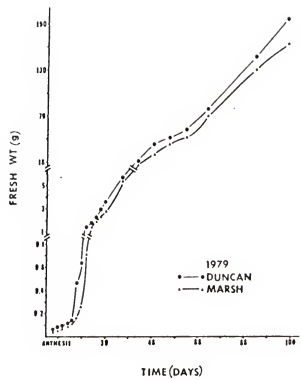
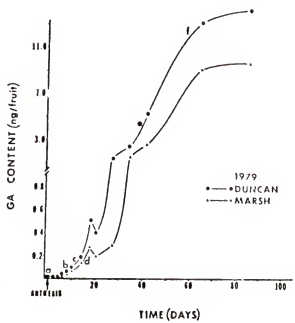


Figure 34. Mean fresh weight of young fruit of 'Duncan' and 'Marsh' grapefruit and seasonal changes in total GA per fruit, 1979. a, Pollination; b, Fertilization; c, Initial division of endosperm; d, Maximum ovule degeneration; e, Division of zygote; f, Cellular endosperm.


FRESH WT (g)
1979
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)


GA CONTENT (ng/fruit)
1979
DUNCAN
MARSH
ANTHESIS
TIME (DAYS)

SUMMARY AND CONCLUSIONS

1. The time of occurrence of various events in the sexual process and the endogenous levels of IAA, GA and ABA for seedy 'Duncan' and seedless (parthenocarpic) 'Marsh' grapefruit from anthesis through early seed development was determined in order to ascertain whether there are any relationships between the sexual process and endogenous levels of these growth substances.

2. Fruit enlargement, as determined by measurements of equatorial diameters, showed both cultivars followed sigmoid growth curves; however, 'Duncan' had attained a slightly larger size when sampling was terminated. The rate of fruit growth on a per day basis showed involvement of IAA in Stage 1 of fruit growth while GA was apparently related to Stage 1 as well as Stage 2.

3. The first peak of fruit drop occurred during and a few days after petal fall and a second, often called the June drop, in late May and early June. No correlation between fruit drop and levels of IAA, GA and ABA was attempted, since critical observations on flower types or of fruit drop were not made. Drop counts were based on both leafy and bouquet bloom while only leafy bloom

samples were used to determine endogenous growth substances.

4. Anatomical results of this study showed 'Duncan' pollen germinated almost immediately on the stigmatic surface and reached the ovule in 8 and 6 days after anthesis in 1978 and 1979, respectively. Actual fertilization was not observed but it must have occurred between the time of entrance of pollen tubes into the ovules and development of free nuclear endosperm, which occurred 4 days later in both seasons. The first evidence of zygote division was observed 31 and 41 days after anthesis in 1978 and 1979, respectively. Pollen grains of seedless 'Marsh' appeared transparent and sterile but an occasional pollen tube as indicated by the presence of callose plugs was observed in the style. There were no observations of entrance of pollen tubes into ovules or further sexual development. Progressive deterioration of ovules in 'Marsh' was consistently observed from anthesis through termination of microscopic examination 16 days after anthesis.

5. Levels of IAA, GA and ABA followed the same pattern in both cultivars and no meaningful differences in their levels were observed. It was evident without the sexual process 'Marsh' fruit produced sufficient growth

substances to promote fruit set while 'Duncan' may have obtained these growth substances from the developing seeds.

6. There are no other reports of levels of IAA, GA and ABA in either 'Duncan' or 'Marsh.' Levels of these growth substances in this study were 21-766 ng IAA, 13-395 ng ABA and 0.062-0.435 ng GA/g fresh fruit weight; whereas those found in other cultivars varied considerably, i.e. 75-690 ng IAA, 125-180 ng ABA and 60.8-150.5 ng GA/g fresh fruit weight.

7. Only part of the sexual process, namely pollination, was related to increases in IAA, GA and ABA, despite the confidence placed in the analytical methods used, as evidenced by repeatability and reproducibility. An overall relationship of levels of endogenous growth regulators and events in the sexual process from pollination to early seed development was not found in 'Duncan.' This conflicts with previous reports that have indicated a direct relationship between the sexual process and growth substances in certain other plants.

8. It is postulated if such a relationship is real, 2 things may have been responsible for lack of an overall relationship. First, levels of growth regulators in tissues that constitute only a small fraction of the

ovary may be related to the sexual process and differences among these tissues might have been obscured in analysis of entire ovaries. Second, many fruit appeared green and healthy in their early stages of growth but turned pale green or yellow and dropped soon thereafter. Thus, fruit sampled may have been predisposed to dropping at the time of sampling due to low levels of one or more growth substances.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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